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(54) Title: ANALYTE INJECTION SYSTEM

(57) Abstract: This invention provides methods and devices for spatially separating at least first and second components in a sample which in one exemplary embodiment comprises introducing the first and second components into a first microfluidic channel of a microfluidic device in a carrier fluid comprising a spacer electrolyte solution and stacking the first and second components by isotachophoresis between a leading electrolyte solution and a trailing electrolyte solution, wherein the spacer electrolyte solution comprises ions which have an intermediate mobility in an electric field between the mobility of the ions present in the leading and trailing electrolyte solutions and wherein the spacer electrolyte solution comprises at least one of the following spacer ions MOPS, MES, Nonanoic acid, D-Glucuronic acid, Acetylsalicyclic acid, 4-Ethoxybenzoic acid, Glutaric acid, 3-Phenylpropionic acid, Phenoxyacetic acid, Cysteine, hippuric acid, p-hydroxyphenylacetic acid, isopropylmalonic acid, itaconic acid, citraconic acid, 3,5-dimethylbenzoic acid, 2,3-dimethylbenzoic acid, p-hydroxycinnamic acid, and 5-br-2,4-dihydroxybenzoic acid, and wherein the first component comprises a DNA-antibody conjugate and the second component comprises a complex of the DNA-antibody conjugate and an analyte.

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ANALYTE INJECTION SYSTEM

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to and benefit of a prior U.S. Provisional Application number 60/532,042, "Analyte Injection System", by Park et al., filed December 23, 2003. The full disclosure of the prior application is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention is in the field of analytical electrophoresis systems and methods. The invention can include high resolution and highly sensitive Isotachophoresis (ITP) and capillary electrophoresis (CE) assays.

BACKGROUND OF THE INVENTION

Electrophoresis is generally the movement of charged molecules in an electric field. Analytical methods based on electrophoresis have found broad utility, especially in the fields of protein and nucleic acid analyses. Samples having charged analyte molecules of interest can be placed in a selective media, such as size exclusion media, ion exchange media, or media having a pH gradient, where they can differentially migrate for high resolution from other sample molecules. The separated molecules can be detected for identification and quantitation.

Capillary and microfluidic scale electrophoretic separations are particularly popular for analyses requiring low sample volumes or high throughput. For example, chips of plastic or glass substrate can be fabricated with microscale loading channels, separation channels and detection channels. Samples can be transferred from microwell plates through a robotically manipulated sample collection tube to the loading channel. An electric potential can induce movement of sample constituents through selective media in the separation channel for sequential detection as the constituents elute into the detection channel from the separation channel. The microscale dimensions of the assay system can provide rapid analyses using microscale, or nanoscale, sample volumes. However, resolution or sensitivity may not be adequate for complex samples or dilute samples.

One approach to enhancing the resolution and sensitivity of capillary electrophoresis (CE) methods has been to pre-resolve and pre-concentrate the sample using Isotachophoresis (ITP) before CE separations. In ITP, the sample is loaded into a channel between a leading

electrolyte (LE) having an electrophoretic mobility greater than the sample and a trailing electrolyte (TE) having electrophoretic mobility less than the sample. Under the influence of an electric field, analytes of interest can migrate through the sample bolus to accumulate at the interface with the LE and/or TE solutions. In this way, the analytes of interest can be separated from certain other constituents of the sample and concentrate to more detectable levels. Samples can thus be concentrated and desalted to provide improved injection material for further capillary electrophoresis separations resulting in highly sensitive detections with high resolution. For example, in "Tandem Isotachophoresis-Zone Electrophoresis via Base-Mediated Destacking for Increased Detection Sensitivity in Microfluidic Systems", by Vreeland, et al., Anal. Chem. (2003) ASAP Article, sample concentrated by ITP is further resolved and detected by capillary zone electrophoresis (CZE). In Vreeland, the sample is subjected to ITP between a TE and an LE having electrophoretic mobilities controlled by the pH of Tris buffers. While ITP concentration of analytes progresses, hydroxyl ions (-OH) are formed by hydrolysis at the cathode end of the separation channel. Migration of the hydroxyl ions through the separation channel eventually neutralizes the Tris buffers to remove the mobility differences between the LE and TE solutions. The Tris neutralization converts the ITP separation media into a CZE separation media. The analytes can then be separated with higher sensitivity and resolution than for standard CZE of the same sample due to the effective sample volume reduction and concentration of analytes resulting from the ITP assay step. The Vreeland method is limited to pH based ITP of compatible samples, can be time consuming due to the neutralization step, and can be inconsistent due to variations in buffer preparation or hydroxyl ion generation.

In another scheme to combine ITP with CE, analytes of interest migrate in ITP mode until they reach an intersection with a CE separation channel before switching the electric field to the separation channel for capillary electrophoresis separation of the analytes. For example, in "Sample Pre-concentration by Isotachophoresis in Microfluidic Devices", by Wainright, et al., J. Chromat. A979 (2002), pp. 69-80, samples are pre-concentrated in a ITP channel until they reach an intersection with a CE channel. The intersection is monitored microscopically by a photomultiplier tube (PMT) receiving light through a confocal lens focused on the intersection. Analytes entering the intersection can be detected, e.g., by fluorescence or light absorption, and the electric field manually switched to inject the analytes into the CE channel. Problems exist, however, in that the manual switching can be inconsistent, some analytes may not be detectable using a PMT, and PMT detection at the microscale can be cumbersome and expensive.

In view of the above, a need exists for increased sensitivity, consistency, and resolution of capillary and microscale electrophoresis methods. It would be desirable to have systems that

can automatically and consistently switch between electrophoretic modes. The present invention provides these and other features that will be apparent upon review of the following.

SUMMARY OF THE INVENTION

The present invention provides, e.g., systems and methods to consistently inject analytes into separation media based on a triggering voltage event. The analytes can be preconditioned and concentrated in a channel by isotachophoresis (ITP) stacking, followed by application of the stacked analytes to a separation channel segment, when a voltage event is detected in the channel.

The methods of the invention can provide highly repeatable analytical results with high sensitivity, speed and resolution. The method can include, e.g., analyte injection by stacking one or more analytes in a stacking channel segment, detecting a voltage potential in the channel, and applying the stacked analyte into a separation channel segment by applying an electric field or a pressure differential along the separation channel segment when a selected voltage event is detected. The channel can be a microscale channel, e.g., with intersecting or common channel segments making up a loading channel segment, a stacking channel segment and/or a separation channel segment.

Stacking of analytes can take place in a stacking channel segment wherein analytes of interest can be sandwiched between buffers selected to focus the analytes into a concentrated band during ITP. Typical injected analytes include, e.g., proteins, nucleic acids, carbohydrates, glycoproteins, ions, and/or the like. The stacking channel segment can have a trailing electrolyte and/or a leading electrolyte which have different mobilities. For example, the leading electrolyte can have a faster mobility under the influence of an electric field than the trailing electrolyte or analytes of interest. In many embodiments, the trailing electrolyte and the leading electrolyte can differ in pH, viscosity, conductivity, size exclusion, ionic strength, ion composition, temperature, and/or other parameters that can affect relative migration or stacking of the electrolytes. The trailing electrolyte can be adjusted to have a mobility less than analytes so that the analytes accumulate at the trailing interface during ITP. Optionally, the leading electrolyte can be adjusted to have a mobility greater than the one or more analytes so that they can accumulate at the leading interface during ITP separations. By narrowly adjusting the migration rates of trailing and leading electrolytes, analytes can be focused between the leading and trailing electrolytes while sample constituents not of interest migrate to other zones of the stacking channel segment. That is, the trailing electrolyte can be adjusted to have a mobility greater than one or more sample constituents not of interest, or the leading electrolyte can be adjusted to have

a mobility less than one or more sample constituents not of interest so that they are not focused with the analyte of interest between the electrolytes.

When the channel of the analyte injection method includes separate stacking and separation channel segments, switching from the stacking channel to the separation channel segment can be by switching the electric field from the stacking channel segment to the separation channel segment, e.g., when the stacked analyte enters an intersection of the stacking and separation channel segments. For example, applying an electric field to the separation channel segment can include switching from a substantial lack of current in the separation channel segment while an electric current flows in the stacking channel segment to an electric current in the separation channel segment while electric current in the stacking channel segment is shut off. Shutting off (substantial lack) of current in the channel segments can be by application of a float voltage to prevent current flow in the channel segment or simply by provision of a high resistance in the channel segment (e.g., allowing no significant electric current outlet from the channel segment). Optionally, switching can be by exerting a pressure differential across the separation channel segment.

The separation channel segment in the injection methods can resolve analytes from other analytes or sample constituents. Such resolution can allow the analytes of interest to be identified or quantitated. Separation channel segments can have selective conditions or separation media to affect migration of analytes and sample constituents. For example, the separation channel can contain a pH gradient, size selective media, ion exchange media, a viscosity enhancing media, hydrophobic media, and/or the like.

Analytes resolved in separation channel segments can be detected for identification and/or quantitation. Detectors can be focused to monitor analytes in the separation channel segment or to detect analytes as they elute from the separation channel segment. Detecting analytes can be by monitoring parameters associated with the analytes, such as, e.g., conductivity, fluorescence, light absorbance, refractive index, and/or the like.

Sample solutions can be loaded to channels of the methods by a variety of techniques, e.g., to provide adequate sensitivity and speed. For example, when the loading channel does not hold enough sample analyte for the desired detection, multiple samples can be consecutively loaded and stacked before fusion of multiple stacks to provide an enhanced concentration of analyte in a small volume. Stacking two or more samples of the analytes can proceed by, e.g.: loading a first sample into a loading channel; applying an electric field across the sample, thereby stacking the sample; loading a second sample into the loading channel; and applying an electric field across the stacked sample and the second sample to stack the second sample and

cause the two stacked samples to become focused together between trailing and leading electrolytes. The multiple stacking technique can be facilitated by flowing the stacked first sample towards the loading channel to clear excess electrolyte and depleted sample solution before loading the second sample. Another way to concentrate sample analytes can be, e.g., by loading samples of the analytes in a loading channel comprising a cross-section greater than a stacking channel segment cross-section so that analytes from a large sample volume do not have to migrate as far to accumulate at a trailing or leading electrolyte interface.

Spacer electrolytes, having migration rates intermediate to two or more analyte species which themselves are intermediate to the trailing electrolyte and the leading electrolyte, can be loaded between samples and/or stacked analytes to resolve the sample into two or more analytes of interest. In one embodiment, stacking comprises loading one or more spacer electrolytes having a mobility greater than at least one analyte species which itself has a higher mobility than the trailing electrolyte and less than at least one other analyte species which itself has a mobility slower than the leading electrolyte between two or more analyte sample segments. In another embodiment, one or more of the two or more analyte sample segments is a previously stacked sample analyte, and the spacer electrolyte is inserted during a multi-stacking load procedure. The spacer may also be included in the sample instead of being injected in between analytes. The spacer electrolytes can be adjusted to provide a mobility between mobilities of two or more of the analytes in order to resolve the analytes in ITP. Such spacer electrolyte adjustments can be made by selecting an appropriate electrolyte pH, spacer electrolyte constituents, spacer electrolyte viscosity, spacer electrolyte conductivity, and/or the like.

In some injection methods, electrolytes can be intelligently formulated to provide ITP resolution of analytes for injection. For example, if the pK of an analyte is determined, e.g., from experiments or calculations, leading and trailing electrolytes can be adjusted to pH values bracketing the pK so that analyte intruding into the leading electrolyte becomes less charged and less mobile, and/or analyte intruding into the trailing electrolyte becomes more charged and more mobile. Such adjustments can enhance the selectivity and concentration of ITP before injection of the stacked analyte.

The injection of stacked analyte into a separation channel segment can be triggered by detection of a selected voltage event. Voltages can be monitored at various locations in the channel and voltage events that precisely indicate preferred timing for injection can be determined. For example, detecting a voltage event can include monitoring a float voltage necessary to maintain a zero current flow (or other defined current flow) condition in the separation channel segment. Typical voltage events used to trigger the start of a separation can

include, e.g., a voltage peak, a voltage trough, a predesignated voltage, relative voltage, absolute magnitude of voltage, derivatives of the voltage as a function of time (e.g. the first derivative measures a rate of voltage change and the second derivative measure the rate of change of the rate of voltage change) (for example a zero slope observed at the top of a voltage profile), time between any of the above events or any combination of the above. The switch to inject stacked analyte from ITP to the separation channel segment can be an automatic application of an electric field or pressure differential along the channel segment when the voltage event is detected.

Systems of the invention for injection of analytes can provide automated injection of stacked analytes for reliable, consistent, and sensitive analyses. Analyte injection systems can include, e.g., an analyte stacking in a channel, a voltage detector in electrical contact with the channel and in communication with a controller so that the controller can initiate a flow of electrical current in a separation segment of the channel, or a pressure differential along the channel segment, when a selected voltage event is detected by the voltage detector. Typically, the channel is a microscale channel having a loading channel segment, a stacking channel segment, and a separation channel segment.

A stacking channel segment in the system is usually configured for isotachophoresis procedures with a trailing electrolyte (TE) and/or a leading electrolyte (LE). The electrolytes can have different adjustable mobilities. For example, the electrolytes can have different pH values, viscosities, conductivities, size exclusion cut-offs, ionic strengths, ion compositions, temperatures, concentrations, or counter and co-ions. Analytes for stacking in the channel can include molecules, such as proteins, nucleic acids, carbohydrates, glycoproteins, derivatized molecules, ions, and the like. Electrolytes can be tailored to selectively stack analytes of interest while rejecting other sample constituents. For example, the trailing electrolyte can be formulated to have a mobility less than the mobility of the analyte of interest and a mobility greater than a mobility of a sample constituent not of interest, so that the analyte accumulates on the front of the TE while the constituent falls away through the TE. The LE can be formulated to have a mobility greater than the mobility of the analyte of interest and a mobility less than a mobility of a sample constituent not of interest, so that the analyte can accumulate at the LE interface while the constituent migrates away in front of the LE interface.

The separation channel segment of the system can contain conditions or selective media to resolve analytes and constituents that have been stacked in the stacking column. For example, the separation column can include a pH gradient, size selective media, ion exchange media, hydrophobic media, viscosity enhancing media, and the like.

The controller can receive output from the voltage detector to initiate an injection when a selected voltage event is detected. The controller can be, e.g., a logic device or a system operator. In some embodiments, the injection event can be a switch from the stacking channel ITP electric field conditions to driving forces required to insert stacked analyte into a separation channel segment. For example, the injection can be a switch from the ITP current flow to substantial elimination of current in the stacking channel segment when the voltage event is detected, while a field or pressure is initiated in the separation channel segment.

The channel segments of the system can include a loading channel segment in fluid contact with the stacking channel segment. Various loading schemes can be employed to meet the demands of particular analyses. In one embodiment, the loading channel segment can have a cross-section greater than a stacking channel segment cross-section so that a larger volume of sample analyte can accumulate in the stacking channel segment in a shorter amount of time, i.e., the average analyte molecule has a shorter migration distance across a large cross-section loading channel segment than with a long loading channel segment of the same volume. In another aspect of loading, a first stacked analyte sample can be pulled back toward the loading channel segment before loading a second sample in a multiple stacking scheme to increase the analyte concentration and sensitivity of an assay. The "pull back" can be accomplished, e.g., by providing a pressure differential across the stacking channel segment to cause the first stacked sample to flow back toward the loading channel segment. Loading channel segments can be filled from, e.g., wells on a microfluidic chip, or by fluid handling systems, such as receiving samples from microarrays through a collector tube (sipper).

Spacer electrolytes can be used in the system, e.g., to enhance resolution between two or more analytes of interest. For example, a spacer electrolyte with a mobility between the mobilities of two or more analytes can be introduced between or with sample segments containing the analytes in the stacking channel segment. Analytes slower than the spacer electrolyte can partition behind the spacer while faster analytes can partition in front of the spacer. In an alternate embodiment, the sample analyte can be combined with spacer electrolytes, e.g., to partition into separate analyte zones, e.g., under the influence of transient or steady state conditions in ITP.

Systems of the invention can have voltage detectors in communication with controllers to detect and respond to voltage events in channels. Voltage detectors can detect voltages between two or more electric contacts across segments of channels, or between contacts at any location in the channel and a voltage reference, such as a ground. In some embodiments of the systems, the voltage detector monitors the voltage in the separation channel segment while stacking

progresses. The voltage of the separation channel segment during stacking can be monitored at an intersection with the stacking channel segment or anywhere along the separation channel segment, e.g., when no substantial current flows in the separation channel segment, such as when a float voltage is being applied to the separation channel segment by a float voltage regulator, where there is no electrical outlet from one end of the channel segment, or where the channel segment has a controlling switch in the off position.

Controllers can automatically switch the system from stacking mode to separation mode on detection of a selected voltage event to inject stacked analytes into the separation channel segment. The voltage event can be, e.g., a voltage peak, a selected voltage, a voltage trough, a relative voltage, a rate of voltage change, and/or the like. The automatic switch can be, e.g., flowing of electrical current in the channel segment, a change in relative voltages across a channel segment, or application of a pressure differential along the channel segment to induce migration of the stacked analytes along the separation channel segment.

Analytes separated in the separation channel segments can be detected by analyte detectors of the system to identify and/or quantitate analytes of interest. Analyte detectors can be configured to monitor analytes in the separation channel segment, or analytes eluting from the separation channel segment. The analyte detector can comprise a fluorometer, a spectrophotometer, a refractometer, a conductivity meter, and/or the like.

The systems of the invention are well suited to microfluidic applications. For example, the loading channel segments, stacking channel segments, separation channel segments, detection chambers, and the like, can be incorporated into a microfluidic chip. The microscale dimensions of microfluidic devices are compatible with many systems of the invention. Microfluidic systems known in the art can provide voltages, pressures, fluid handling, communications, and detectors, etc., useful in practicing the systems of the present invention.

DEFINITIONS

Unless otherwise defined herein or below in the remainder of the specification, all technical and scientific terms used herein have meanings commonly understood by those of ordinary skill in the art to which the present invention belongs.

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular methods or systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example,

reference to "a constituent" can include a combination of two or more constituents; reference to "the analytes" can include one analyte, and the like.

Although many methods and materials similar, modified, or equivalent to those described herein can be used in the practice of the present invention without undue experimentation, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

The term "analyte", as used herein, refers to constituents of a sample that are detected by an analyte detector. An "analyte of interest", as used herein, refers to an analyte for which detection and/or quantitation is desired in an assay.

The term "channel", as used herein, refers to a conduit for flowing and/or retention of fluids in methods and systems of the invention. Channels can be, e.g., tubes, columns, capillaries, microfluidic channels, and/or the like. A channel can include various channel segments, e.g., in separate sections of the channel, that share sections of the channel, and/or that intersect with other segments of the channel. Channel segments are generally functional sections of channel, such as, e.g., loading channel segments, stacking channel segments, and separation channel segments.

A "skewing channel" in the invention can be a channel segment that causes skewing of sample constituents flowing in the channel. For example, the internal surface topography of a skewing channel can cause bands or peaks to take on an oblique orientation relative to the channel axis while passing through the skewing channel.

The term "mobility", as used herein, refers to a rate of migration for charged molecules, such as analytes or electrolytes, in a solution under the influence of an electric field in a channel.

The term "float voltage", as used herein, refers to a voltage required in a channel segment to substantially prevent flow of an electric current through the segment or to establish a desired constant current in the segment.

The term "microscale", as used herein, refers to dimensions ranging from about 1000 μm to about 0.1 μm.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of an isotachophoresis system.

Figure 2 is a schematic diagram of transient ITP concentrating an analyte at an interface with a leading electrolyte.

Figure 3 is a schematic diagram of transient ITP separation of analytes of interest and steady state ITP juxtaposition of the analytes.

Figure 4 is a schematic diagram of selective removal of sample constituents during ITP.

Figures 5A to 5C are schematic diagrams of exemplary sample solution loading techniques.

Figures 6A to 6E are sequential schematic diagrams describing a technique of stacking multiple loads of sample analytes.

Figures 7A to 7C are schematic diagrams showing enhanced sample solution volume loading using a loading channel segment with a cross-section greater than the cross section of the stacking channel segment.

Figures 8A to 8D are schematic diagrams of voltage event detection at a contact point in a stacking channel segment.

Figures 9A to 9D are schematic diagrams of analyte band skewing caused by flow through a skewing channel.

Figures 10A and 10D are schematic diagrams of sample constituent skewing and dispersion in skewing channel ITP while an analyte of interest band remains focused.

Figures 11A to 11C are schematic diagrams of stacked analyte application to a separation channel segment.

Figures 12A and 12B are schematic diagrams of a microfluidic chip with a collector tube feeding sample solutions to a loading channel segment.

Figures 13A and 13B are schematic diagrams of an analyte injection system wherein a stacking channel segment shares a common channel with a separation channel segment.

Figures 14A and 14C are schematic diagrams of an analyte injection system incorporating skewing channels in spiral and serpentine configurations.

Figure 15 is a schematic diagram of a skewing channel with an increased ratio of outside travel distance over inside travel distance through a turn.

Figures 16A and 16C are schematic diagrams of a skewing channel with skewing provided by providing a travel surface distance on one side greater than for the other side of the channel.

Figure 17 is a schematic diagram of an exemplary microfluidic chip channel configuration useful for performing isotachophoresis using spacer molecules and for separating and isolating a component peak of interest from an undesirable component peak according to an alternative embodiment of the invention.

Figures 18A-D are schematic diagrams of a a portion of the channel configuration of Figure 17 which is useful for separating the component peak of interest from an undesirable

component peak and for separating the component peak of interest into separated components and detecting the separated components.

Figure 19 is an alternative channel configuration to that shown in Figures 18A-D which is useful for separating and isolating the component peak of interest from an undesirable component peak and for separating the component peak of interest into separated components and detecting the separated components

Figure 20A shows the voltage and optical signature of a DNA-antibody conjugate and antigen complex which are separated from one another using isotachophoresis with appropriate spacer molecules; Figures 20B-C are exploded views of the DNA antibody conjugate peak (Figure 20B) and antigen complex peak (Figure 20C) shown in Figure 20A showing the voltage slope transitions which occur within about one-half second of the detection of the optimal maxima signal profiles for the respective component peaks.

Figures 21 show an exemplary voltage and optical signature of a DNA-antibody conjugate and antigen complex during the performance of an immunoassay for the detection of AFP levels in serum which shows that there are at least three voltage slope transitions which can be used to trigger the switchover from the isotachophoresis stacking phase to the CE separation phase of the assay.

DETAILED DESCRIPTION

The invention relates to methods and systems for injection of analytes into separation channels. Stacking sample analytes can provide higher analyte concentrations in smaller injection volumes for electrophoretic separations with improved assay sensitivity and resolution. Sensitivity and separations can be improved, in many cases, by stacking analytes in skewing channels before injection. Automated timing of injections triggered by detection of voltage events can improve the consistency of results between assay runs.

Methods and systems of the invention can be used to separate, identify, and/or quantify analytes with a high level of sensitivity and resolution. Analytes of the invention can be, e.g., charged molecules, such as, e.g., proteins, nucleic acids, carbohydrates, glycoproteins, ions, derivitized molecules, and/or the like.

METHODS OF ANALYTE INJECTION

Methods of the invention can provide precise injection timing of stacked analyte into a separation channel for sensitive, repeatable, high resolution assays. Methods of the invention generally include, e.g., loading a sample to a loading channel segment before isotachophoresis (ITP) in a stacking channel segment, detecting a voltage event that indicates a stacked sample

analyte is in position for injection, applying an electric field or pressure differential to apply the stacked sample analyte to a separation channel segment, and detecting separated analytes of interest. The ITP can include migration of the analytes through skewing channels. Detection signals can be evaluated to determine the presence or quantity of the analytes.

Stacking Analytes of Interest

Analytes of interest can be stacked into a volume less than the original analyte sample by isotachophoresis (ITP). For example, a sample bolus can be loaded between two different buffer systems in a channel and exposed to an electric current to create a steady state of solute zones migrating in order of decreasing mobility. In the steady state, the zones can adopt the same concentration and migrate along the channel at the same velocity as the leading electrolyte. Alternatively, a sample bolus can be loaded adjacent to an electrolyte and stacked in a dynamic (transient) condition at the interface for injection, e.g., without having reached a steady state equilibrium between ITP electrolytes.

Stacking can be practiced, e.g., in channels of a microfluidic chip wherein a sample is loaded between channel regions of a trailing electrolyte and a leading electrolyte. As shown in Figure 1A, analyte sample **10** can be loaded to loading channel segment **11** by a differential pressure between vacuum wells **12** and sample well **13**. When an electric field is applied across stacking channel segment **14**, current is carried by high mobility (e.g., high charge to mass ratio) leading electrolytes **15**, intermediate mobility analytes **16**, and low mobility trailing electrolyte **17**, as shown in Figure 1B. As ITP proceeds, a steady state can be established in which the volume of analyte **16** is reduced to the point where the concentration of charged analyte **16** is equivalent to the concentration leading electrolyte **15**. In the steady state, the stacked analyte solution migrates along stacking channel segment **14** at the same rate as the leading and trailing electrolytes, as shown in Figure 1C, with the electrolytes and charged analytes carrying the same amount of electric current per unit volume in the stacking channel segment. Factors, such as charge density and transient differential migration rates of the analytes and electrolytes, tend to focus the analytes and electrolytes into zones during ITP. Stacking channel segments of the invention can be any size including microscale channels having a dimension, such as width or depth, ranging from about 1000 μm to about 0.1 μm, or from about 100 μm to about 1 μm, or about 10 μm.

Stacking can also be practiced in a transient state. For example, as shown in Figure 2A, initially dilute and dispersed analyte molecules **20** can accumulate, e.g., at leading electrolyte interface **21** as shown in Figure 2B. This concentration of analyte at an interface can occur before establishment of steady state uniform analyte and electrolyte carrier concentrations.

Optionally, an analyte can accumulate in a transient state, e.g., during initial application of an electric field in ITP, at trailing electrolyte interface **22**. In other embodiments or transient ITP, analytes can become concentrated in zones other than interfaces of ITP electrolytes.

Multiple analytes of interest can accumulate in a steady state or transient state, e.g., at one or both of the electrolyte interfaces. For example, as shown in Figures 3A to 3C, sample solution **30** with first analyte of interest **31** and second analyte of interest **32** can be loaded between trailing electrolyte solution **33** and leading electrolyte solution **34**. In the case where the first analyte has a slower mobility than the second analyte, but a faster mobility than the trailing electrolyte, the first analyte can accumulate at the interface with the trailing electrolyte in the presence of an electric field. Meanwhile, in the transient state, as shown in Figure 3B, the second analyte, with somewhat higher mobility than the first analyte, can accumulate at the other end of the sample bolus along the interface with the faster mobility leading electrolyte. Such a situation can provide the opportunity for separate sequential or parallel application of the first and second analytes to one or more separation channel segments, as can be appreciated by those skilled in the art. Once a steady state has been established in the ITP, as shown in Figure 3C, charged first and second analytes can become compressed into narrow adjacent bands, e.g., for application together for resolution in a separation channel segment.

In methods of the invention, the mobilities of trailing electrolytes and leading electrolytes can be adjusted to provide selective pre-concentration of an analyte of interest while separating sample constituents not of interest from the analyte. For example, as shown in Figure 4A, sample solution **40** containing analyte of interest **41**, slow mobility sample constituent not of interest **42**, and fast mobility sample constituent not of interest **43**, can be loaded between trailing electrolyte **44** and leading electrolyte **45**. When an electric field is applied to the channel, slow mobility sample constituents not of interest **42** can fall behind the trailing electrolytes while fast mobility sample constituent not of interest **43** can race ahead of the leading electrolytes, as shown in Figure 4B. Continued ITP to a steady state can, e.g., further separate sample constituents not of interest from the analyte, as shown in Figure 4C. Removal of sample constituents not of interest from analytes of interest can provide an improved injection material for separation in a separation channel segment. After samples have been pretreated by ITP to remove sample constituents not of interest, analyses of analytes of interest applied to a separation channel segment can have, e.g., reduced background noise, higher resolution due to lower injection volumes, more accurate quantitations due to better baselines and fewer overlapping peaks, etc.

Trailing electrolytes and leading electrolytes can be tailored, according to methods known in the art, by adjusting electrolyte mobilities to provide highly specific retention and stacking of analytes of interest, while sample constituents not of interest are removed. In one embodiment of the methods, the pH of electrolytes is selected to bracket the pK of an analyte of interest so that sample constituents not of interest having pKs outside the bracket will be removed in the ITP. The pK of the analytes of interest can be determined, e.g., empirically or based on the known molecular structure of the analytes. In other embodiments, the analyte of interest can be, e.g., closely bracketed between selected trailing and leading electrolyte compositions known to have slower and faster mobilities than the analyte. Many ions and buffers can be used in electrolytes to bracket analytes, such as, e.g., chloride, TAPS, MOPS, and HEPES. Optionally, the mobility of electrolytes and/or analytes can be modulated by adjusting the viscosity or size exclusion characteristics of the sample solution, trailing electrolyte solution, and/or leading electrolyte solution. In another option for adjusting the mobility of ITP solutions, mobility of analyte solutions and/or electrolyte solutions can be moderated, particularly during transient ITP migrations, by adjusting the concentration, ionic strength, or conductivity of the solutions. The temperature of solutions can be selected in still other options to adjust the mobility of analytes, electrolytes, or ITP solutions.

A variety of sample solution loading methods can benefit analyses in methods of the invention. Stacking channels can be loaded with single sample solution loads, with multiple sample solution loads, and with spacer electrolyte between sample solution loads, as described in detail below.

Single sample loads can be loaded to sample loading channel segments according to techniques known in the art, e.g., as shown in Figures 5A to 5C. Sample solution **50** can be applied to loading channel segment **51** using, e.g., electroosmotic flow (EOF) or a differential pressure to flow the sample solution from sample well **52** through the loading channel segment and out through waste channel **53** intersecting and offset along the loading channel segment, as shown in Figure 5A. Alternately, Sample solution **50** can be loaded to branch into loading channel segment **51** under the influence of a differential pressure between sample well **52** and waste wells **54** as shown in Figure 5B. In figures 5A and 5B, the pressures in other wells with no flow must be adjusted to ensure zero flows. In another sample loading alternative, a relative vacuum at waste wells **54** can draw sample solution **50**, the trailing electrolyte, and the leading electrolyte in a "pinching" flow, as shown in Figure 5C, for precise and consistent definition of sample volumes.

Additional amounts of sample solution can be loaded for ITP using a multiple stacking technique. A first sample can be loaded into loading channel segment **60** as shown in Figure 6A. An electric field can be applied across stacking channel segment **61** to stack sample analytes **62**, as shown in Figure 6B. The stacked sample analytes **62** can be flowed back towards the loading channel segment and second load of sample solution **63** loaded adjacent to the first stacked analytes, as shown in Figure 6C. An electric field can be applied across the stacking channel segment a second time to stack the second sample analytes **64**, as shown in Figure 6D. Separation zone **65**, substantially composed of trailing buffer, can exist initially during the second stacking, but can dissipate as trailing electrolytes fall behind the second stack analytes in the electric field. Eventually, the first and second stacked analytes can combine under the influence of the electric field to form multiple stack **66** having, e.g., twice the amount of analytes as the first stack, as shown in Figure 6E. The amount of analyte in the multiple stack can be further increased by additional rounds of stack pull back, sample loading, and stacking.

Optionally, a large volume of sample solution can be loaded into a loading channel segment having a cross-section greater than the cross-section of the stacking channel segment. As shown in Figure 7A, sample solution **70** can be loaded into large cross-section loading channel segment **71**, e.g., with a differential pressure across sample well **72** and waste well **73**. Under the influence of an electric field, sample analytes **74** can be concentrated near the stacking channel segment entrance, as shown in Figure 7B. Loading channel segments with increased cross section can concentrate analytes in a shorter time due to the reduced axial distance **75** for analyte travel as compared to a similar volume loading channel segment with a smaller cross section. Trailing electrolyte **76** can optionally be brought to a position adjacent to concentrated sample analytes **74** for subsequent ITP by, e.g., providing a pressure differential to flush the loading channel segment with trailing electrolyte, e.g., as shown in Figure 7C.

Advantages can be obtained in methods of the invention by placing a spacer electrolyte between analyte sample segments for ITP. The spacer electrolyte can have a mobility intermediate between the trailing electrolyte and the leading electrolyte. The spacer electrolyte can have a mobility intermediate between two or more analytes of interest. The spacer electrolyte can provide, e.g., enhanced resolution between multiple analytes of interest. In one embodiment, spacer electrolyte can be present in loaded sample solutions to provide a spacer zone between analytes on application of an electric field. In another embodiment, spacer electrolyte can be loaded between cycles of multiple stacking. For example, multiple stacking can proceed as described above, but with spacer electrolyte present to the left of the initial stack, with spacer electrolyte present in one or more loaded sample solution segments, or by loading

spacer electrolyte between cycles of loading sample solution segments. Spacer electrolytes can be adjusted as described above for adjustment of trailing and leading electrolyte mobilities to tailor spacer migration between analytes of interest.

Detecting Voltage Events

Detection of voltage events associated with, e.g., migration of solutions, analytes, and/or electrolytes in the stacking channel segment can provide, e.g., a consistent signal for initiation of stacked analyte application to a separation channel segment. During an ITP, voltage potentials across the stacking channel segment, or voltages measurable at any point along the stacking channel segment, can vary with time. From one ITP run to the next, there can be measurable voltage events that are consistent between runs and which can act as timing markers useful for consistent triggering of injections and the switch from an ITP to a different separation scheme.

In a typical embodiment of detecting a voltage event, trailing electrolyte, analyte, and leading electrolyte are flowing in a stacking channel segment during an ITP. The trailing electrolyte has a higher resistance to electric current flow than the leading electrolyte. With a voltmeter monitoring voltage, e.g., at a point half way along the stacking channel segment, as shown in Figure 8, voltage events can be detected as the ITP proceeds. With sample solution **80** initially loaded and applied to the stacking column entrance, leading electrolyte **81** fills the stacking channel segment and the voltage detected at contact **82** half way along the channel segment is about half the ITP electric field voltage. As the analyte and trailing electrolyte **83** migrate down the stacking channel segment, resistance increases on the entrance side of the stacking channel segment resulting in a detectable voltage rise at the voltmeter contact, as shown in Figure 8B. At about the time stacked analyte reaches the point of voltmeter contact, the difference in electrical resistance on the two sides of the point of contact reaches a maximum along with the detected voltage, as shown in Figure 8C. Finally, as the analyte approaches the end of the stacking channel segment, now substantially filled with trailing electrolyte, the resistance on both sides of the contact equalize and detected voltage returns to about half the ITP electric field voltage, as shown in Figure 8D. Voltage events, in this example could include the starting voltage value, the start of voltage rise, the rates of changes (slope, concavity etc.) of the voltage rise or fall, the maximum voltage (voltage peak), the slope of zero observed at maximum voltage, the return to starting voltage, any predetermined voltage, any relative voltage between locations in the channel segments, time between two or more of any of the example events and/or the like. Consistent, but somewhat different, voltage profiles can be observed, e.g., with one or more voltmeter contacts located at different points along the stacking channel segment. These consistent measurable voltage events can be selected, e.g., to trigger switches in electric

current or pressure differentials in channel segments to apply stacked analytes to a separation channel segment.

A separation channel segment in electrical contact with a stacking channel segment will have no substantial flow of electric current if the separation channel is not part of a complete circuit (e.g., a "dead end" with no ground connection) or if a float voltage is applied to the separation channel segment. In a preferred configuration for detecting voltage events, the voltmeter contact can be located at a point between the separation channel segment and the stacking channel segment, or at any location along the separation channel segment. In one preferred embodiment, voltage events can be detected by monitoring a separation channel segment float voltage.

Enhancing Separations in Skewing Channels

Separation of analytes of interest from other sample constituents can be enhanced by stacking the analyte during and/or after passage through a skewing channel segment. For example, sensitivity of an assay can be increased when sample constituents not of interest become dispersed by the turns while the analyte of interest continues to be focused by electrolytes in the isotachophoresis method.

Analyte bands flowing in channels of an analytical system can become dispersed when the channel diverges from a straight path. For example, as shown in Figures 9A to 9D, analyte **90** flowing on the inside of turn **91** travels a shorter distance than analyte flowing on the outside of the turn. The initially compact band can become skewed and dispersed along a greater length of the channel, as shown in Figure 9C. Axial diffusion of the skewed band can dilute the band and prevent realignment of the band, as shown in Figure 9D. A detector focused on the band in Figure 9A would detect a stronger and narrower maximum signal for the band than a detector focused on the band in Figure 9D after skewing and diffusion. Such dispersion of bands can be problematic an many chromatographic analysis because of the resultant broadening and shortening of peaks. However, the present invention can combine, e.g., intentionally accentuated skewing with ITP technology to enhance separations by stacking analytes of interest while dispersing sample constituents not of interest.

In one embodiment, for example, a small amount of analyte of interest can be separated from a larger amount of sample constituent not of interest with an enhanced degree of sensitivity and improved quantitation. In an ITP system without skewing channels, as shown schematically for example in Figure 10A, a small amount of stacking analyte of interest **100** can migrate, e.g., between selected trailing and leading electrolytes, while a larger amount of sample constituent not of interest **101**, with a mobility similar to the trailing electrolyte, migrates near the front of

the trailing electrolyte. Detector **102** focused on the channel can fail to resolve the analyte and sample constituent peaks, as shown in detector output signal chart **103**. The analyte sensitivity and quantitation capabilities can be enhanced by, e.g., introducing one of more skewing channel segments into the stacking channel. Analyte **100** and sample constituent **101** migrating in the staking channel (Figure 10B) can become skewed and dispersed in skewing channel **104** (Figure 10C). Some time after exiting the skewing channel, the staking forces of the leading and trailing electrolytes can focus and realign the analyte peak in the channel, while the un-shepherded sample constituent peak remains skewed and becomes diffused. A detector focused on the channel can detect the presence and quantity of analyte against a diminished and less intrusive background of sample constituent.

The benefits of ITP separations in skewing channels can be increased by selecting trailing and/or leading electrolytes to enhance the stacking focus of the analyte while increasing the mobility difference between the electrolytes and the sample constituent. In selective ITP the mobilities of leading and trailing electrolytes are selected, e.g., to be near the known mobility of an analyte and/or to increase the difference in mobility between the electrolytes and one or more sample constituents not of interest. For example, in the situation described above, where the analyte of interest has a mobility greater than the sample constituent not of interest, the trailing electrolyte can be selected to have a mobility closer to the analyte than to the sample constituent so that, e.g., the analyte is closely shepherded while the sample constituent lags behind to experience the effects of skewing and diffusion. In a similar fashion, if the analyte of interest has a mobility less than the sample constituent not of interest, the mobility of the leading electrolyte can be selected to be between the analyte mobility and the sample constituent mobility to enhance skewing channel ITP separation. In a preferred embodiment, the mobility of an electrolyte is selected to be between the mobilities of the analyte of interest and one or more sample constituents not of interest but closer to the mobility of the analyte. In another example, both leading and trailing electrolytes can be selected to be close to the known mobility of the analyte of interest. This can provide particular benefits when both faster and slower sample constituents migrate near the analyte and/or when transient stacking prevails during the ITP.

The effectiveness of skewing channel ITP can vary widely depending on factors, such as, e.g., the radius of any turns involved, the internal diameter of the channel, the topography of the channel walls, the cross section of the skewing channel, the flow velocity, and the viscosity of solutions. For example, as is discussed in the Skewing Channel ITP Systems section below, skewing in a channel can be increased with short turn radii, repeated turns in the same direction, channel topographies that increase the difference between the surface length of opposite channel

walls, and channel cross sections that are wider perpendicular to the axis of a turn. Appropriate conditions for a particular method or system can be derived, e.g., through calculation and/or experimentation.

To consider how diffusion can affect the amount of skew caused by a turn, a two-dimensional, nondimensionalized advection-diffusion equation can be considered (see also, Analytical Chemistry, vol 73, No. 6, 1350-1360, March 15, 2001):

$$\frac{\partial c'}{\partial t'} + \underbrace{u'\frac{\partial c'}{\partial x'}}_{\text{advection}} = \frac{1}{Pe'_w}\left[\underbrace{\frac{w}{L}\left(\frac{\partial^2 c'}{\partial x'^2}\right)}_{\text{axial diffusion}} + \underbrace{\frac{L}{w}\left(\frac{\partial^2 c'}{\partial y'^2}\right)}_{\text{transverse diffusion}}\right]$$

wherein L is the length of the turning channel, w is the internal width of the turning channel, and Pe'_w is the dispersion Peclet number; u', c', t', x' and y' are the normalized velocity, concentration, time, axial channel dimension, and transverse channel dimension, respectively. Three parameters, Pe'_w, L, and w, have been determined to be of special importance to skewing and dispersion of analytes under the influence of skewing channels in the present invention.

The Peclet number (Pe) is a dimensionless factor representing a ratio of advection (or forward movement) and diffusion of an analyte. If Pe is large, peaks skewed by passage through a first skewing channel can retain a stable oblique shape long enough to have it reversed by a second turn in the opposite direction. If Pe is small, peaks skewed in a skewing channel can diffuse across the width of the channel in a relatively short time to convert a skewed peak into a diffusely broadened peak. In methods of the invention, sample constituents not of interest can be most readily skewed and dispersed from analytes of interest, e.g., when conditions exist in skewing channels providing a Peclet number more than about the ratio of the length of the skewing channel over the internal width of the skewing channel (i.e., $Pe > L/w$). Significant benefits in skewing, diffusion, and dispersion of sample constituents not of interest in skewing channel ITP can be obtained where conditions provide a Peclet number more than about 0.01times, 0.1 times, 1 time, 10 times, 100 times, or more, than the ratio of the skewing channel length over the skewing channel width.

Conditions affecting the Peclet number can be, e.g., conditions that influence advection and/or diffusion of molecules in the channels, as is known by those skilled in the art. For example, Pe can be influenced by the viscosity of solutions, the presence of a gel, temperature, molecular concentrations, the velocity of the molecule along the channel, the diameter of the channel, and/or the like. Adjustment of conditions controlling advection and diffusion can

provide Peclet numbers, e.g., that result in desirable levels of sample constituent dispersion during and/or after passage through skewing channel segments of the invention.

Applying Stacked Analytes to Separation Channels

Analytes stacked by ITP can be injected into a separation channel segment, e.g., by applying an electric field or pressure differential across the separation channel segment and the stacked analytes. The field and/or pressure can cause migration or flow of analytes into the separation channel segment. Application of the field or pressure can be triggered by detection of a voltage event, as described above, to provide consistent and functional analyte injection timing. Application of the separation channel segment electric field or pressure differential can coincide with elimination of current flow in the stacking channel segment. The timing between the voltage event and the injection can be established to conform to particular configurations of channels, intersections, and solution segments. The timing can also play a key role in determining the peak resolution and signal strength as it can affect the amount of transient isotachophoresis that persists after the handoff.

Separation channel segments can provide conditions for electrophoretic separation of analytes and/or separation by selective media. In preferred embodiments, separation channel segments have a microscale dimension (e.g., a depth or width ranging from about 1000 μm to about 0.1μm, or from about 100 μm to about 1 μm), e.g., to provide fast separations of small analyte sample volumes. Separation channel segments can have separation media, such as, e.g., a pH gradient, size selective media, ion exchange media, a viscosity enhancing media, hydrophobic media, and/or the like, capable of contributing to the resolution of analytes. Separation channel segments (as well as stacking channel segments) can have viscosity enhancing media, such as gels, to reduce electroosmotic flow (EOF) in separation modes where EOF is undesirable. Separation channel segments can be independent from other channel segments, or can share all or part of a channel with other channel segments, such as, e.g., loading channel segments and stacking channel segments. In a preferred embodiment, the separation channel segment is independent, but intersects in a fluid contact at some point along the length of the stacking channel segment.

In a typical embodiment, stacked analyte from an ITP separation is injected into a separation channel segment when a peak voltage is detected at the intersection of a stacking channel segment and the separation channel segment. For example, the float voltage in separation channel segment **110** reaches a maximum (and the rate of voltage change, or slope of the voltage profile, becomes zero) as stacked analytes **111**, sandwiched between trailing electrolyte **112** and leading electrolyte **113**, migrate in an ITP past a voltmeter contact at the

intersection of the separation channel segment with the stacking channel segment, as shown in Figure 11A. The voltage maximum can trigger the elimination of the ITP electric field in the stacking channel segment and the application of an electrophoresis electric field in the separation channel segment to induce migration (application) of stacked analytes **111** into the separation channel segment, as shown in Figure 11B. Migration of analytes through selective media of the separation channel segment can separate (resolve) analytes of interest **114** from sample constituents not of interest **115** that co-migrated with the analytes through the stacking channel segment during ITP, as shown in Figure 11C. In some embodiments, multiple analytes of interest that stacked together, or in proximity to each other, during ITP can be resolved from each other in the separation channel segment, e.g., by capillary zone electrophoresis.

Alternate schemes for timing of injection will be appreciated by those skilled in the art. Such alternate schemes can be based, e.g., on calculations or models, or can be determined empirically. For example, time delays can be built into triggered responses based on channel volumes, channel geometry, voltmeter contact location, choice of voltage events, the location of analytes relative to solution features affecting voltage events, and/or the like. In a particular example, wherein analyte is stacked near a trailing electrolyte interface in a transient ITP (not yet reaching a steady state) and the remaining sample solution bolus has a high electrical resistance, a suitable trigger time can be a certain time after the voltage peak to allow the stacked analyte additional migration time to reach the intersection with the separation channel segment.

Application of an electric field along the separation channel segment can be automatic (that is, not requiring manual switching). Such automatic application of the electric field can be accomplished, e.g., by electronic devices and algorithms known in the art. For example, a voltmeter can be set to trip a switch when voltage at a contact reaches a set level. In preferred embodiments, a logic device, such as, e.g., an integrated circuit or a computer, can be programmed to initiate switching of actuators according to preset parameters (e.g., the occurrence of defined voltage events).

Detecting Analytes

Analytes separated in by methods of the invention can be detected in the separation channel segment and/or sequentially after elution from the separation channel segment. Appropriate detectors can, e.g., be fixed to monitor analytes in a detection channel, sequentially scan for analytes in channel segments, or provide continuous imaging of entire channels.

Appropriate detectors are often determined by the type of analyte to be detected. Proteins and nucleic acids, for example, can often be detected by spectrophotometric monitoring of particular light absorption wavelengths. Many ionic analytes of interest can be detected by

monitoring changes in solution conductivity. Many analytes are fluorescent or can be labeled with fluorescent markers for detection using a fluorometer. Many analytes in solution, particularly carbohydrates, can be detected by refractometry.

In a typical embodiment, detecting can be by monitoring transmission of a light source through a separation channel segment using a photomultiplier tube (PMT) focused on the channel with a microscope lens. Those skilled in the art will appreciate how such an arrangement can be configured as a fluorescence detector by addition of an appropriate excitation light source, such as, e.g., a laser or filtered light from a lamp. Optionally, the lens can be mounted on an X-Y scanning mechanism to monitor any location on a microfluidic chip. With such an arrangement, the length of a separation channel segment can be scanned for analytes, e.g., resolved along a pH gradient. In another embodiment, conductivity meter sensors can be mounted across a separation channel outlet to monitor charged analytes as they elute from the channel segment.

Detectors can be in communication with data storage devices and/or logic devices to document assay runs. Analog output from detectors, such as PMTs and conductivity meters, can be fed to chart plotters to retain a trace of the analyte separation profile on paper. Analog to digital converters can communicate detection signals to logic devices for data storage, separation profile presentation, and/or assay evaluation. Digital logic devices can greatly facilitate quantitation of analytes by comparison to appropriate standard curves from regression analysis.

ANALYTE INJECTION SYSTEMS

Electrokinetic analyte injection systems described herein can provide sensitive analyte detection with high resolution in a highly consistent manner. Analytes selectively stacked in a stacking channel segment can be injected (applied) into a separation channel segment with precise timing based on detection of voltage events in the channels. Such precision can be enhanced by provision of automated injection subsystems.

Systems of the invention generally include, e.g., an analyte stacking in a channel, a voltage detector in communication with a controller and in contact with the channel at one or more locations, an electric current or pressure differential established in the channel when a selected voltage event is detected by the voltage detector and communicated to the controller. The channel can include stacking channel segments and separation channel segments that intersect, form a continuous channel or which share common channel sections. Analytes applied to the separation channel segment and separated can be, e.g., detected by a detector in

communication with a logic device to determine the presence of particular analytes or to evaluate the quantity of analytes.

Channels

The channel of the invention can be, e.g., a single multifunction channel comprising loading segments, stacking segments, separation segments, and/or detection segments. Optionally, the channel can include separate loading channel segments, stacking channel segments, and separation channel segments in fluid contact at intersections. In a preferred embodiment, as shown schematically in Figure 11, the loading channel segment is an extension of the stacking channel segment, and the separation channel segment is in fluid contact with the stacking channel segment through an intersection where analyte injection takes place. Channels of the systems can be any known in the art, such as, e.g., tubes, columns, capillaries, microfluidic channels, and/or the like. In a preferred embodiment, the channels are microscale channels, e.g., on a microfluidic chip.

Channels of a microfluidic device can be embedded on the surface of a substrate by mold injection, photolithography, etching, laser ablation, and the like. The channels can have a microscale dimension, such as, e.g., a depth or width ranging from about 1000 μm to about 0.1 μm, or from about 100 μm to about 1 μm. Fluids can flow in the channels, e.g., by electroosmotic flow, capillary action (surface tension), pressure differentials, gravity, and/or the like. Channels can terminate, e.g., in wells of solutions and/or at intersections with other channels or chambers. Channels can have electrical contacts, e.g., at each end to provide electric fields and/or electric currents to separate analytes or to induce EOF. Detectors can be functionally associated with channels to monitor parameters of interest, such as, e.g., voltages, conductivity, resistance, capacitance, electric currents, refractivity, light absorbance, fluorescence, pressures, flow rates, and/or the like. Microfluidic chips can have functional information communication connections and utility connections to supporting instrumentation, such as electric power connections, vacuum sources, pneumatic pressure sources, hydraulic pressure sources, analog and digital communication lines, optic fibers, etc.

The channel can include, e.g., a load channel segment to introduce one or more sample solution volumes into the channel. Such loading channels can be configured in ways appreciated by those skilled in the art, such as, e.g., as an injector loop, to include an a collector tube **120** to a microfluidic chip, as shown in Figure 12, and/or as a flushed channel segment, as shown schematically in Figures 5A to 5C. Loading channel segments can have a cross-section greater than the cross-section of the stacking channel segment, as shown in Figure 7, to provide rapid

concentration of analytes near the stacking channel segment entrance from a large volume of sample solution.

Channels of the systems can contain gelatinous substances to beneficially affect migration and flow characteristics of the channels as described, for example, in United States Patent Application Ser. No. 60/500,177 for "Reduction of Migration Shift Assay Interference," filed on September 4, 2003, the entire contents of which are incorporated by reference herein. Gels can be incorporated into channels to reduce unwanted electroosmotic flows of solutions while providing a more electrophoretic character to a separation. Gels can influence the relative migration rates of analytes and/or electrolytes by slowing the progress of larger molecules. Gels can provide tools to help adjust migration zones for analytes and ITP electrolytes in stacking channel segments. For example, analytes of interest are generally larger than commonly used ITP electrolytes. By placing a gel in a stacking channel segment, a fast analyte (large but with a high charge to mass ratio) can be slowed to migrate behind a leading electrolyte small molecule salt or buffer. Optionally, a gel can slow an analyte to migrate only marginally faster than a trailing electrolyte. Gel resistance to large molecule migration can be adjustable, e.g., by altering the type of gel, concentration of gel matrix, and the extent of gel matrix cross-linking. Gels can provide enhanced concentration and/or resolution to analytes in stacking or separation channel segments. One or more different gels can be present in either the stacking channel segment or the separation channel segment. A variety of different gels can be used in practicing the methods of the present invention including without limitation polyacrylamide gel, polyethylene glycol (PEG), polyethyleneoxide (PEO), a co-polymer of sucrose and epichlorohydrin, polyvinylpyrrolidone (PVP), hydroxyethylcellulose (HEC), poly-N,N-dimethylacrylamide (pDMA), or an agarose gel. The gel is preferably present in the microfluidic channel(s) of the device at a concentration of between about 0.1 and 3.0%, for example between about 0.9 and 1.5%.

Stacking channel segments can function to selectively stack analytes of interest, e.g., by ITP, for injection into a separation channel segment for further resolution and detection. Stacking channel segments can have electrical contacts, e.g., at each end, for application of electric fields suitable for analyte stacking. Stacking channel segments can have fluid contacts with, e.g., externally driven pneumatic or hydraulic manifolds so that pressure driven flows, such as electrolyte loading or the pull back for the multiple stacking technique discussed in the "Stacking Analytes of Interest" section above, can be practiced. Stacking channel segments can contain, e.g., electrolytes, such as trailing electrolytes, spacer electrolytes, and/or leading electrolytes, suitable for isotachophoresis (ITP), as discussed in the Methods section above. The

stacking channel segment can have trailing electrolyte well **18**, as shown in Figure 1, and leading electrolyte well **19**, for introduction of electrolytes into channel segments.

Separation channel segments can receive stacked analytes by injection from stacking channel segments for further resolution by separation techniques, such as, e.g., additional rounds of ITP, ion exchange, size exclusion, hydrophobic interaction, reverse phase chromatography, isoelectric focusing, capillary zone electrophoresis, and/or the like. Separation channel segments can include electric contacts for application of electric fields along the channel segment and/or external connections with pressure sources to drive fluid flows. Separation channel segments can be, e.g., a channel segment intersecting a stacking channel segment, a channel segment continuing a common channel with a stacking channel segment, and/or a channel segment functionally sharing channel sections with a stacking channel segment. In a typical embodiment, the separation channel segment intersects the stacking channel segment at some point along the stacking channel segment length, as shown in Figure 11. In this embodiment, sample constituents not of interest can remain in separate stacking channel segment sections after injection of stacked analytes of interest into the separation channel segment. In other embodiments, e.g., the stacking and separation channel segments can functionally reside in a common channel without an intervening intersection. For example, e.g., as shown in Figure 13A, stacking can continue in a channel segment until a voltage event is detected. On detection of the voltage event, conditions can change in the channel for a transition to a separation mode. Such a transition can include, e.g., application of a differential pressure between channel ends **130** to induce analyte flow into size exclusion resin **131**, as shown in Figure 13B. Smaller molecules will elute past detector **132** before larger molecules. Other examples of transitions to separation modes can include, e.g., changes in the direction of electric current flow, changes in the direction of fluid flow, injections of separation buffers into a channel, changes in an electric field voltage, and/or the like.

Skewing Channel ITP Systems

Isotachophoresis systems of the invention can include skewing channel segments in, and/or before, the stacking channel to enhance the separation of analytes of interest from sample constituents not of interest. The sample constituents can be dispersed while the analyte of interest is focused by stacking, e.g., in the skewing channels. The separation enhancement can be promoted, e.g., by turning through cumulatively large angles, sharp turning, skewing channel cross sections having relatively large widths, skewing channel topographies with opposite surfaces of different length, and/or skewing channel systems having conditions providing a

Peclet number more than about the ratio of the skewing channel length over the skewing channel width.

One way to increase skew and dispersion in skewing channel segments is to provide greater turning angles in the channel. In a two dimensional plane, turning angles can be accumulated, e.g., with continuous spiral turns or switching serpentine turns as shown in Figures 14A to 14 C. Spiral turns have the advantage that turning angles can accumulate through a large number of degrees in one direction, with a concomitant accumulation of skew. A disadvantage of spiral skewing channels can be the inherent continuous expansion of the turn radius into a range of less effective curvatures. Spiral skewing channel configurations can also entail difficult access problems for connections to the inner channel end. One way to provide accessible channel ends in a spiral skewing channel configuration can be to have side by side spiraling channels running in and out of the center, as shown in Figure 14B. Alternately, the access to a spiral channel end can be provided in the third dimension, e.g., through a sipper tube or a back channel in another plane, e.g., as shown in Figure 14A. Another limitation on the length of the spiral channel is that the Peclet number required for optimal skewing increases as the length of the spiral channel increases. Serpentine skewing channels, as shown in Figure 14C, can provide easy access to channel ends but complimentary turns can cancel the skew of previous turns, particularly where the Peclet number is large or the time is short between turns. Optionally, three dimensional skewing channels can be employed, such as helices and coils.

Skew and dispersion from passage through skewing channel segments can be more pronounced in channels that make sharp turns relative to the internal channel diameter. For example, skew is increased for skewing channels with a high ratio of channel internal diameter over turn width. In one embodiment, the skew from a skewing channel segment having turns is increased when the cross-section of the channel is greater along the radius of the turn (skewing channel internal width) than perpendicular to the turn radius (skewing channel depth).

The topography of a skewing channel segment can affect the skew and dispersion of migrating analytes. For example, channel surface contours that increase the ratio between the travel distance along the outside of a turn over the travel distance along the inside of the turn can increase skew. Skew can be increased by increasing channel internal width relative to channel depth at turn points. As shown in Figure 15, analyte **150** can become highly skewed by flowing through a turn having a bolbus outer turn surface. Skew can be enhanced in skewing channels where the travel surface distance on a first side **151** of the skewing channel is greater than the travel surface distance on a second side **152** of the skewing channel, even if there is no curvature in the skewing channel overall, as shown in Figure 16. For example, significant skewing can be

provided from differences in opposite surface travel distances ranging from more than about 500%, to 100%, to 50%, to 10%, or less.

Selective stacking of the analyte of interest between leading and trailing electrolytes is an important aspect of skewing channel ITP systems of the invention. Analytes of interest can be continuously refocused between the electrolytes during and/or after skewing while sample constituents not of interest become dispersed. The mobility of an analyte of interest can be known from calculations or by empirical data. The trailing and/or leading electrolyte can be selected to have a mobility between those of the analyte of interest and intrusive sample constituents not of interest. To enhance focusing of the analyte and dispersion of sample constituents, the electrolytes can be selected to have mobilities closer to that of the analyte of interest than the sample constituents.

Skewing ITP channel segments can be incorporated into the systems and methods of injecting analytes described above. An analyte of interest can be injected into a separation channel at higher purity after dispersion of other sample constituents by skewing channel ITP. Injection of the analyte can be initiated on detection of a voltage event.

ITP with Spacer Molecules and Isolation of Sample Component Peaks

The present invention provides additional techniques for improving the sharpness of resolution of the isotachophoresis systems of the invention. These additional techniques may find particular applicability when using the teachings of the present invention for mobility shift immunoassays using fluorescently labeled antibody conjugates as described, for example, in co-pending patent application USSN 60/500,177 entitled "Reduction of Migration Shift Assay Interference," filed on September 4, 2003. Migration shift immunoassays are useful methods to detect and quantify associations between biomolecules. A change in the retention time of a molecule in an electrophoretic or chromatographic assay, for example, can indicate the presence of a binding molecule. Binding can be specific, such as in the case of antibody-antigen interactions, or non-specific, such as the ionic attraction of a positively charged molecule to a negatively charged polymer.

Migration shifts can be observed in other interactions of affinity molecules with analytes. Migration shifts can be observed, for example, when an antibody binds to an antigen, or when a polysaccharide binds to a lectin. However, chromatography or electrophoresis of these molecules often provides broad and poorly resolved peaks due to multiple conformations and unstable charge density in these molecules. The diversity of possible affinity molecule/analyte pairs can also require development of a special migration shift assay for each pair. These problems can be avoided if the affinity molecule is linked to a carrier polymer that is highly

resolved in assays under a standard set of conditions. An example of technology using a carrier/affinity molecule conjugate is described, e.g., in Japanese Patent Application number WO 02/082083, "Method for Electrophoresis", which is hereby incorporated by reference in its entirety. Although use of uniform carrier molecules for affinity molecules in migration shift analyses can improve resolution, a problem remains with interference from excess labeled antibody conjugate peaks, especially when a large excess of labeled antibody conjugate is used to accelerate the kinetics of the binding reaction and to improve the dynamic range of the assay. For example, a problem that arises with adding excess labeled antibody conjugate is that it often creates a large peak in the electrophoretic separation pattern, and this large peak can interfere with detection of the antigen bound conjugate (i.e., antigen complex) that is used to detect the presence of the antigen or analyte in the sample.

A need therefore remains for methods to block or substantially eliminate the interference from excess labeled conjugate migration peaks in migration shift assays, particularly in assays utilizing affinity molecule carriers. Several techniques are described in the art that may be used to address this problem, such as the addition of a second antibody conjugate to the binding reaction mixture that further shifts the mobility of the antigen complex away from the antibody conjugate peak, as is described, e.g., in U.S. Patent No. 5,948,231, the entire contents of which are incorporated by reference herein. In addition, the use of isotachophoresis techniques with spacer molecules of intermediate mobilities between the leading and trailing electrolyte ions can provide further spacing between the antibody conjugate and antigen complex peaks to help improve the resolution of those peaks as described, for example, in Kopwillem, A. et al., "Serum Protein Fractionation by Isotachophoresis Using Amino Acid Spacers," J. Chroma. (1976) 118:35-46 and Svendsen, P.J. et al., "Separation of Proteins Using Ampholine Carrier Ampholytes as Buffer and Spacer Ions in an Isotachophoresis System," Science Tools, the KLB Instrument Journal (1970) 17:13-17, the entire contents of which are each incorporated by reference herein.

However, even when using such techniques, it has been found that when large concentrations of labeled antibody conjugate are used and small amounts of analyte (e.g., antigen) are present in a sample (e.g., on the order of about 1 picomolar or less), such as a complex human serum sample, that the antibody conjugate migration peak may still tend to disperse into the region of the antigen complex thereby affecting the detection sensitivity of the assay.

The teachings of the present invention described herein can be used to substantially eliminate the antibody conjugate source of interference by separating the conjugate away from

the antigen complex prior to injecting the complex into the separation channel of a microfluidic device (e.g., where the antigen complex is separated from other contaminating components in the sample). In particular, as described further below, a method of separating a first component of interest (e.g., an antigen complex) from at least a second component (e.g., excess labeled antibody conjugate) in a sample (e.g., a clinical sample derived from a body fluid or tissue sample) is disclosed which generally comprises stacking the first and second components in a first channel segment by isotachophoresis; flowing the stacked second component through a second channel segment fluidly coupled to the first channel segment at an intersection, detecting a preselected electrical signal at or near the intersection which corresponds to either the first and/or the second stacked component; and applying an electric field or a pressure differential along a third channel segment which is fluidly coupled to the intersection when the preselected electrical signal is detected, thereby introducing the stacked first component into the third channel segment. The method may further comprise separating the stacked first component into separated components in the third channel segment, and detecting the separated components.

In one particular embodiment the stacking comprises introducing into the first channel segment a leading electrolyte buffer, a trailing electrolyte buffer, and a spacer buffer solution having spacer molecules with an electrophoretic mobility intermediate an electrophoretic mobility of the leading and trailing electrolyte ions, and stacking the first and second components by isotachophoresis. The leading electrolyte may be selected, for example, from the group comprising salts of chloride, bromide, fluoride, phosphate, acetate, nitrate and cacodylate. The trailing electrolyte may be selected, for example, from the group comprising HEPES, TAPS, MOPS (3-(4-mor-pholinyl)-1-propanesulfonic acid), CHES (2-(cyclohexylamino) ethanesulfonic acid), MES (2-(4-morpholinyl)ethanesulfonic acid), glycine, alanine, beta.-alanine and the like. The spacer molecule may be selected, for example, from the group comprising MOPS (3-(4-mor-pholinyl)-1-propanesulfonic acid), Ampholine, an amino acid, MES, Nonanoic acid, D-Glucuronic acid, Acetylsalicyclic acid, 4-Ethoxybenzoic acid, Glutaric acid, 3-Phenylpropionic acid, Phenoxyacetic acid, Cysteine, hippuric acid, p-hydroxyphenylacetic acid, isopropylmalonic acid, itaconic acid, citraconic acid, 3,5-dimethylbenzoic acid, 2,3-dimethylbenzoic acid, p-hydroxycinnamic acid, and 5-br-2,4-dihydroxybenzoic acid, or any other appropriate spacer that comprises ions that have an electrophoretic mobility that is between the electrophoretic mobilities of the ions present in the leading and trailing electrolyte buffers. The spacer molecules provide a separation region between the stacked first component and the stacked second component at the ion fronts between the spacer molecules and the leading and trailing electrolytes.

Isotachophoresis of the sample can be performed by generating an electric potential across the first and second channel segments to cause the second component to stack and then flow into the second channel segment (where it is isolated from the first stacked component of interest). As described above, the first component can comprise, for example, a fluorescently labeled antigen-antibody complex and the second component can comprise a fluorescently labeled antibody (e.g., a labeled DNA-antibody conjugate). The first and second components are preferably both charged, wherein the first and second components may both be negatively charged or may both be positively charged, or one component may be positively charged and the other negatively charged. The first and second charged components may also be selected, for example, from the group comprising nucleic acids, proteins, polypeptides, polysaccharides, and synthetic polymers.

The step of detecting an electrical signal may comprise, for example, detecting an optical signal, a voltage signal, or a current signal at or near the intersection of the first and second channel segments. Thus, by using a combination of isotachophoresis spacer molecules and a microfluidic channel network design and assay script that allows one to trap unwanted component migration peaks in a side channel isolated from the main separation channel, it has been found that the sharpness of resolution obtainable by isotachophoresis can be substantially improved.

With reference now to Figure 17, a schematic diagram of one exemplary microfluidic chip channel configuration useful for performing isotachophoresis using spacer molecules and for separating and isolating a component peak of interest from an undesirable component peak is shown. The microfluidic chip of Figure 17 contains a channel network generally designated **150** which includes a number of channels or channel segments, several of which terminate in a buffer or electrolyte reservoir. Specifically, the channel network includes channel segment **162** which terminates in a trailing electrolyte buffer reservoir **160**, channel segment **166** which terminates in a waste reservoir **168**, channel segment **172** which terminates in a sample (and spacer buffer) reservoir **174** which contains a spacer buffer such as MOPS, channel segment **178** which terminates in waste reservoir **180**, a short interconnecting channel segment **184** at the fluid junction of ITP stacking channel segment **182** and separation channel segment **194** which further junctions into channel segments **186** and **190** which in turn terminate in spacer buffer reservoir **188** and leading electrolyte buffer reservoir **192**, respectively, and channel segments **196** and **200** which terminate in leading electrolyte buffer reservoirs **198** and **202**, respectively. Note that the composition of the respective buffer reservoirs may vary depending on the particular uses of the microfluidic chip. The leading electrolyte reservoirs **192**, **198**, and **202** are filled with a solution

of an electrolyte having ions with a higher electrophoretic mobility than the mobilities of any of the sample components. The trailing electrolyte reservoir **160** is filled with a solution of an electrolyte having ions with a lower electrophoretic mobility than the mobilities of any of the sample components. The spacer buffer reservoirs **174** and **188** are filled with a solution of an electrolyte having ions with an electrophoretic mobility in an electric field intermediate that of the leading and trailing electrolytes. The sample, which in this case, is placed into spacer buffer reservoir **174**, contains at least two different sample components, e.g., a DNA antibody conjugate and an antigen-DNA-antibody complex.

The microfluidic chip also includes a number of connecting channel segments **164**, **170**, and **176**, and ITP stacking channel segment **182** and separation channel segment **194** fluidly coupled thereto, which complete the overall channel network. The reservoirs of the chip are adapted to be coupled to either a vacuum (or pressure) source and/or adapted to receive an electrode, or both. Examples of multi-port pressure control microfluidic devices and systems which include means for selectively and independently varying pressures and/or voltages within the reservoirs of the system can be found, for example, in co-pending patent application USSN 09/792,435 entitled "Multi-Port Pressure Control Systems," filed February 23, 2001, the entire contents of which are incorporated by reference herein. Where used, the electrodes, when placed in appropriate reservoirs, may be formed on the substrate or formed independently, e.g., on an electrode plate for placement on the substrate for electrode contact with liquid in the associated reservoirs. Each electrode, in turn, is operatively coupled to a control unit or voltage controller (not shown) to control output voltage (or current) to the various electrodes. A vacuum or pressure source (not shown) is also provided to supply an appropriate vacuum (or pressure) to one or more of the associated reservoirs. A multi-reservoir pressure controller can be coupled to a plurality of independently controlled pressure modulators to effect pressure-based movement of fluids within the channels of the microfluidic channel network, as described in co-pending patent application USSN 09/792,435 referenced above. By selectively controlling and changing the pressure applied to the reservoirs of the microfluidic device, hydrodynamic flow may be accurately controlled at desired flow rates within intersecting microfluidic channels. The pressure-induced flows may be combined with electrokinetic fluid control thereby providing a composite pressure/electrokinetic based flow control system useful for loading a sample into the channels of the system and for performing ITP based assays according to the teachings of the present invention. Although only a single channel network is shown in Figure 17, it is to be appreciated that the device may include an array of channel networks, each having the general features of the above-described channel network.

To load a sample into the channel network to perform the initial sample stacking step using isotachophoresis with spacer molecules, it is preferable to load the sample using pressure-induced flow control to help decrease any sample biasing effects caused by the electrical fields associated with electrokinetic fluid transport. However, it is to be understood that the sample loading technique described herein may also rely on electrokinetic fluid control and transport as necessary (e.g., where the system is not equipped with a multi-port pressure control capability). A vacuum is first applied to waste reservoirs **168** and **180**, while a corresponding counter-pressure (or vacuum) is applied to reservoir **188** to inhibit the flow of spacer buffer solution into channel segment **186**. The application of a vacuum to reservoirs **168** and **180** will cause terminating electrolyte **160** to flow into and fill channel segment **164**, while the sample which is placed in spacer buffer reservoir **174** will flow into and fill channel segments **170** and **176**. In addition, leading electrolyte from buffer reservoirs **192**, **198** and **202** will flow into and fill channel segments **182** and **194**. Thus, such a flow pattern will position the sample and spacer buffer solution sandwiched between the trailing electrolyte solution in channel segment **164** and the leading electrolyte buffer solution in channel segments **182** and **194**.

To cause the sample to stack into two (or more) small volumes (e.g., corresponding to the DNA antibody conjugate and antigen complex in the sample) by ITP, a positive voltage gradient is then established between electrodes in fluidic contact with reservoirs **160** and **192**, which will cause ITP to occur in channel segments **170**, **176** and the main stacking channel segment **182**.as the sample moves through those respective channel segments. The spacer buffer (designated "SP" in Figures 18A-D) provides a separation region between the two stacked volumes **210** and **212** in the sample, e.g., in this case between the stacked antibody conjugate peak **210** and the stacked antigen complex peak **212**, at the ion fronts between the spacer and the leading and trailing electrolyte buffer solutions (designated "L" and "T", respectively, in Figures 18A-D). This is best illustrated in Figures 18A-D and Figure 19. The antibody conjugate peak **210**, which travels faster than the antigen complex peak **212**, is allowed to migrate first into the side channel **184** and towards reservoir **192** via channel segment **190**. A voltage detector (e.g. voltmeter) and/or an optical detector is/are placed into sensory communication with the intersection **187** of channel segments **188** and **192**, to monitor the voltage signature and/or optical signal of the sample as it passes the intersection **187**. As used herein, the phrase "in sensory communication" refers to a detection system that is positioned to receive a particular signal from a particular location, e.g., a microscale channel. For example, in the case of optical detectors, sensory communication refers to a detector that is disposed adjacent a transparent region of the microscale channel or fluid intersection or junction in question, and configured such that an

optical signal from the channel, e.g., fluorescence, chemiluminescence, etc., is received and detected by the optical detector. Such configuration typically includes the use of an appropriate objective lens and optical train positioned in sufficient proximity to the fluidic element or channel to gather detectable levels of the optical signal. Microscope based detectors, e.g., fluorescence detectors are well known in the art. See, e.g., U.S. Pat. Nos. 5,274,240 and 5,091,652, each of which is incorporated herein by reference.

As noted above, when a peak voltage is detected at the intersection **187** which corresponds to the stacked second component **210**, the detected voltage signature can trigger through appropriate process means the elimination of the ITP electric field generated between reservoirs **160** and **192**, and the subsequent application of an capillary electrophoresis (CE) electric field in the separation channel segment **194** to induce migration (application) of stacked first component peak **212** into the separation channel segment, as shown in Figures 18C-D. In order to time the switching of the voltage gradient as described above, voltage, current and/or optical signal data from the intersection **187** (or from the fluid junction intersection of the ITP stacking channel segment **182** and separation channel segment **194**, e.g., for the chip configuration of Figure 19) can be used. Based on such data as described below, the voltage gradient can then be switched to be between reservoirs **188** and **202**, while allowing the electrode in contact with reservoir **192** to float such that there is no current in channel segment **190**.

Figures 20A-C show an exemplary voltage and optical signature of a DNA-antibody conjugate and antigen complex which were separated from one another using isotachophoresis with appropriate spacer molecules and a microfluidic channel network similar to that of Figure 17. As shown, the components in the sample produce two optical maxima **216** and **218**, respectively, and two voltage slope changes in the voltage signal **220** and **222**, respectively. The optical maxima signals **216**, **218** and the occurrence of the subsequent voltage slope changes **220**, **222** occur within about one-half of a second of each other, as best seen in Figures 20B-C. In other words, the first voltage slope change **220** occurs about ½ second after the occurrence of the first optical maximum **216**, and the second voltage slope change **222** occurs about ½ second after the occurrence of the second optical maximum **218**. Thus, the measurement of the occurrence of either one of the voltage slope changes **220**, **222** (and/or the optical signal maxima **216**, **218**) can be used to signal the switchover of the voltage gradient change to wells **188** and **202** for the separation phase of the assay from wells **160** and **192** for the ITP phase of the assay. By controlling the relative conductivities of the buffers and spacers, it is possible to control the magnitude of the voltage slope changes to make the above measurements easier to detect.

With reference to Figure 21, it has also been observed that in certain assay configurations that the voltage (and optical signal profile) includes more than two, e.g., three or more, distinct voltage slope changes which occur relatively close in time to one another (e.g., on the order of about ½ second or less). This has been shown to be the situation for the performance of immunoassays in microfluidic devices for the detection of Alpha-fetoprotein (AFP), which is an early fetal plasma protein, the functional equivalent of albumin, which is produced by the fetal yolk sac, liver, and gastrointestinal tract as described, for example, in co-pending U.S. Application Ser. No. 60/500,177 for "Reduction of Migration Shift Assay Interference," filed on September 4, 2003, which has previously been incorporated by reference herein. In the case of an AFP immunoassay, it is often necessary to distinguish and compare different levels of various fractions of AFP. AFP has been shown to be divided into at least 3 fractions through the lectin-affinity electrophoresis using lens culinaris agglutin (LCA). LCA separates AFP into three bands: LCA-non-reactive (AFP-L1), weakly reactive (AFP-L2); and strongly reactive (AFP-L3). A relative comparison of the levels of AFP L1 to AFP L3, for example, has been shown to be useful as a marker for hepatocellular carcinoma and total AFP as a marker in pregnant women for the potential occurrence of neural tube defects in children. In the performance of an AFP immunoassay using a DNA-antibody conjugate to capture the various AFP fractions of interest in a microfluidic system as described in more detail in USSN 60/500,177 noted above, although any one of the voltage slope changes can be used to signal the switchover of the voltage gradient change to wells **188** and **202** for the CE separation phase of the assay from wells **160** and **192**, it has been observed that the use of the last-in-time voltage derivative (e.g., third distinct voltage change **224** shown in Figure 21 for each of the four patent samples run through the device) provides the optimum results in triggering that switchover.

Migration of the first stacked component of interest **212** through the separation channel segment **194** can separate (resolve) components of interest **214** in the sample by capillary zone electrophoresis, as shown in Figure 18D. By introducing spacer buffer into the separation channel segment **194** via reservoir **188** and channel segment **186** (and **184**), the first stacked component **212** will be sandwiched between spacer buffer solutions at both its upstream and downstream fluid boundaries, which will cause the de-stacking and separation of the stacked component **212** from any other contaminating species that were stacked during the ITP phase of the assay in ITP stacking channel segment **182**.

Because it may not be possible to divert all of the stacked second component **210** into channel segment **184**, leading to some carryover of stacked component **210** in the separation channel segment **194**, the presence of spacer buffer as the trailing buffer in the separation

channel segment will mean that any undesirable carryover component material **210** in the separation channel will be sandwiched between a slower spacer buffer and a faster leading electrolyte buffer which will cause it to further stack in the separation channel segment **194**. The self-sharpening properties of this ITP interface will thus minimize interference caused by the presence of carryover second component **210** in the separation channel segment and diffusion of the faster moving component **210** into the slower moving component peak **212**. In this way, a substantial amount of the component **210** and any other labeled materials not of interest that are stacked with the component of interest **212** (e.g., antigen complex) and which could interfere with the mobility shift assay are substantially separated from the stacked component **212**. This can significantly reduce the amount of materials that affect the baseline signal in the detection region of the separation channel segment and thus improves the sensitivity of the assay. It is to be noted that the presence of sieving media in the various buffer solutions can assist in regulating the mobility of the components of interest during the ITP phase of the assay, and can also improve the separation of contaminating species from the component **212** during the CE phase of the assay.

To further minimize possible interference from any carryover of the conjugate peak into the separation channel segment **194**, an alternative embodiment of a channel network configuration can be employed as shown in Figure 19 in which the presence of an interconnecting channel segment **184** connecting channel segments **186** and **190** to the fluid junction of the ITP stacking channel segment **182** and the separation channel segment **194,** is eliminated. In this alternative embodiment, the voltage detector and/or optical detector would be placed into sensory communication with the fluid junction between channel ITP stacking channel segments **182** and separation channel segment **194**. In addition, in this particular embodiment, the detection of the second voltage slope change **222** (or second optical maximum **218**) corresponding to the component peak of interest **212** would be used to trigger the switch of the voltage gradient from the ITP phase of the assay to the CE phase of the assay in the separation channel segment **194**, ensuring that almost all of second component **210** enters the side channel **190** where it is discarded to fluid reservoir **192**. In further alternative embodiment of the invention, channel segment **186** could also intersect with and be positioned on the opposite side of channel segments **182, 194** from that of channel segment **190**.

Voltage Detectors

Voltage detectors in systems of the invention can be in contact with channels to detect voltage events communicated to a controller. The type and complexity of voltage detectors can depend on, e.g., channel hardware configurations and the type of voltage event to be detected.

Voltage detectors can range from, e.g., simple relay switches tripped by a voltage, to analog galvanometers, to analog devices with chart recorders, to voltmeters with digital outputs for evaluation by logic devices. Voltmeters generally detect a voltage potential between electrodes at two locations, such as, e.g., a contact location in a channel and a ground, or between two different locations in a channel. The location of the voltage electrode contacts with the channel can change the voltage profile detected during a stacking run. However, a well defined voltage event can often be determined for consistent and unambiguous triggering of an injection for voltmeter contacts at a wide range of channel locations (e.g., the voltmeter contact does not have to be at an intersection between stacking and separation channel segments).

In one embodiment, voltmeter contacts can be located at two ends of the channel. As trailing electrolyte, of relatively high resistance, displaces leading electrolyte in the channel, the voltage required to maintain a selected current through the channel can increase. A voltage event to trigger injection in this case can be, e.g., a preset voltage.

In another embodiment, voltmeter contacts can be located at a ground (or other voltage reference) and at any point in a separation channel segment intersecting a stacking channel segment. If electric current is not allowed to flow through the separation channel segment (e.g., where the separation channel segment is held at zero current by a float voltage, or where the separation channel segment not part of a complete circuit), any location in the separation channel segment will reflect the stacking channel segment voltage at the intersection. Voltage detected in the separation channel segment can rise to a peak and fall as the TE/LE interface passes the intersection, in a fashion similar to the voltage profile of Figure 8, as will be appreciated by those skilled in the art.

Where voltage is being monitored in a separation channel segment without electrical current and in contact with the stacking channel segment, the lack of current can be by, e.g., float voltage regulation or circuit isolation. A float voltage regulator device can be an electronic device, known in the art, that detects electric current flow in a channel segment and applies a voltage to the channel segment that neutralizes any voltage potential across the channel segment, thus preventing a flow of electric current. A float voltage regulator can optionally be configured to adjust a channel segment voltage differential to provide a selected constant current in the channel segment. Another way to prevent electric current flow in a channel segment is to ensure that the channel segment is not a part of a completed electric circuit. For example, an electric switch can be present at one end of the channel segment to selectively open or close any associated electric circuits.

The voltmeter can communicate with a controller for initiation of analyte application (injection) to a separation channel segment. Initiation of injection can be manual or automatic. For example, the voltmeter can provide a visible voltage readout for a system operator (the controller) to manually switch channel electric fields or fluid flows on observation of a voltage event, such as a selected voltage or voltage peak. In another example, the controller is a digital logic device in electronic communication with the voltmeter and set to automatically apply stacked analytes to a separation channel segment on detection of a selected voltage event.

Analyte Detectors

Appropriate analyte detectors can be incorporated into systems of the invention to detect analytes. The type and configuration of detectors can depend, e.g., on the type of analyte to be detected and/or on the layout of channels. Analyte detectors can be in communication with logic devices for storage of analyte detection profiles and evaluation of analytical results.

Analytes for detection in the systems can range widely, with many being charged molecules or molecules modified to have a charge. For example, analytes of interest can be proteins, nucleic acids, carbohydrates, glycoproteins, ions, and/or the like. Although stacking can take place by alternate mechanisms, such as size exclusion, stacking is driven by migration of charged analytes in an electric field for many systems of the invention. It will be appreciated by those skilled in the art that non charged analytes of interest can receive a charge for electrophoretic stacking by appropriate adjustment of pH or derivatization of the analyte with a charged chemical group.

Analyte detectors in the systems can be any suitable detectors known in the art. For example, the detectors can be fluorometers, spectrophotometers, refractometers, conductivity meters, and/or the like. Analytes not detectable by available detectors can often be derivitized with a marker molecule to render then detectable. The detectors can be mounted or focused to monitor analytes in the channel segments, including, e.g., intersections and/or separation channel segments. Detectors can monitor analytes as they exit separation channel segments, e.g., in detection channels of chambers.

Analyte detectors can monitor a channel location, sequentially scan a channel length, or provide a continuous image of separated analytes. In one embodiment, a stationary spectrophotometric detector can be a photomultiplier tube focused on a particular channel location or intersection. In another embodiment, the analyte detector can be a fluorometer focused on microchannels through a confocal microscope lens mounted to an X-Y transporter mechanism to sequentially scan analytes separated in channels of a microfluidic device. In

another embodiment, the analyte detector can be a charge coupled device (CCD) array capable of providing an image of numerous separations in multiple separation chambers at once.

The analyte detector can be in communication with a logic device for storage and evaluation of analytical results. Logic devices of the systems can include, e.g., chart recorders, transistors, circuit boards, integrated circuits, central processing units, computer monitors, computer systems, computer networks, and/or the like. Computer systems can include, e.g., digital computer hardware with data sets and instruction sets entered into a software system. The computer can be in communication with the detector for evaluation of the presence, identity, quantity, and/or location of an analyte. The computer can be, e.g., a PC (Intel x86 or Pentium chip- compatible with DOS®, OS2®, WINDOWS® operating systems) a MACINTOSH®, Power PC, or SUN® work station (compatible with a LINUX or UNIX operating system) or other commercially available computer which is known to one of skill. Software for interpretation of sensor signals or to monitor detection signals is available, or can easily be constructed by one of skill using a standard programming language such as Visualbasic, Fortran, Basic, Java, or the like. A computer logic system can, e.g., receive input from system operators designating sample identifications and initiating analysis, command robotic systems to transfer the samples to the loading channel segments of the system, control fluid handling systems, control detector monitoring, receive detector signals, prepare regression curves from standard sample results, determine analyte quantity, and/or store analytical results.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, many of the techniques and apparatus described above can be used in various combinations.

All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

1. A method of separating a first component of interest from at least one second component in a sample comprising:

stacking the first and second components in a first channel segment;

flowing the stacked second component through a second channel segment fluidly coupled to the first channel segment at an intersection;

detecting a preselected electrical signal at or near the intersection which corresponds to the first and/or second stacked component; and,

applying an electric field or a pressure differential along a third channel segment which is fluidly coupled to said first channel segment at the intersection when the preselected electrical signal is detected, thereby introducing the stacked first component into the third channel segment.

2. The method of claim 1, wherein said stacking comprises introducing into the first channel segment a leading electrolyte buffer solution, a trailing electrolyte buffer solution, and a spacer buffer solution between the leading and trailing electrolyte solutions wherein the spacer buffer solution comprises ions which have an intermediate mobility in an electric filed between the mobility of the ions present in the leading and trailing electrolyte solutions.

3. The method of claim 1, further comprising separating the stacked first component into separated components in the third channel segment.

4. The method of claim 1, wherein the first and third channel segment comprise channel portions of a single, contiguous channel.

5. The method of claim 1, wherein said flowing step comprises generating an electric potential across said first and second channel segments to cause said stacked second component to flow into said second channel segment.

6. The method of claim 1, wherein said first component comprises a flourescently labeled antigen-antibody complex and said second component comprises a fluorescently labeled antibody.

7. The method of claim 1, wherein said first and second components are both charged.

8. The method of claim 1, wherein said first and second components are both negatively charged or both positively charged.

9. The method of claim 1, wherein at least one of said first and second components is positively charged.

10. The method of claim 7, wherein said first and second charged components are selected from the group comprising nucleic acids, proteins, polypeptides, polysaccharides, and synthetic polymers.

11. The method of claim 7, wherein the first and second charged components comprise labeled molecules having distinct electrophoretic mobilities.

12. The method of claim 3, further comprising detecting said separated components.

13. The method of claim 1, wherein said sample is a clinical sample derived from a body fluid or tissue sample.

14. The method of claim 2, wherein said leading electrolyte is selected from the group comprising salts of chloride, bromide, fluoride, phosphate, acetate, nitrate and cacodylate.

15. The method of claim 2, wherein said trailing electrolyte is selected from the group comprising HEPES, TAPS, MOPS (3-(4-mor-pholinyl)-1-propanesulfonic acid), CHES (2-(cyclohexylamino) ethanesulfonic acid), MES (2-(4-morpholinyl)ethanesulfonic acid), glycine, alanine, and .beta.-alanine.

16. The method of claim 2, wherein the spacer buffer solution comprises ions which have an intermediate mobility in an electric filed between a mobility of the first and second components.

17. The method of claim 16, wherein said second component comprises a DNA-antibody conjugate and said first component comprises a complex of the DNA-antibody conjugate and an analyte.

18. The method of claim 1, wherein said detecting an electrical signal comprises detecting an optical signal.

19. The method of claim 1, wherein said detecting an electrical signal comprises detecting a voltage signal.

20. The method of claim 1, wherein said detecting an electrical signal comprises detecting a current signal.

21. The method of claim 1, wherein said second channel segment is fluidly coupled to the intersection via an interconnecting channel segment which intersects with the first channel segment at one end and intersects the second channel segment at its other end, wherein the detecting a preselected electrical signal at or near the intersection comprises detecting the preselected electrical signal at the intersection of the second channel segment with the interconnecting channel segment.

22. A method of separating a first component of interest in a sample into separated components and detecting the separated components while minimizing interference during detecting from at least one second component in the sample, the method comprising introducing the sample into a separation channel and applying an electric field along a length of the separation channel to separate the first component of interest into separated components according to their electrophoretic mobilities while concomitantly stacking the second component in the separation channel between a leading electrolyte and a trailing electrolyte solution, and detecting the separated components.

23. The method of claim 21, wherein the trailing electrolyte solution comprises a spacer buffer solution, and wherein the first component of interest is sandwiched between spacer buffer solutions on both sides of the first component in the separation channel.

24. A microfluidic device comprising a main channel comprising an ITP stacking channel region and a separation channel region; and at least first and second side channels which are fluidly coupled to the main channel at a common fluid junction at the intersection of the ITP stacking channel region with the separation channel region, the first and second side channels terminating in first and second fluid reservoirs, respectively.

25. The microfluidic device of claim 24, wherein the first fluid reservoir is filled with a spacer buffer solution and the second fluid reservoir is filled with a leading electrolyte solution.

26. The microfluidic device of claim 24, wherein the first and second side channels both intersect the main channel at the common fluid junction.

27. The microfluidic device of claim 24, further comprising a connecting channel which intersects with the common fluid junction at one end and intersects with the first and second side channels at its other end.

28. The microfluidic device of claim 24, wherein the common fluid junction includes a detection region which is configured to be located in sensory communication with a voltage detector and/or an optical detector.

29. The microfluidic device of claim 28, wherein the voltage detector and/or optical detector is configured to detect an electrical signal from a first stacked component and/or a second stacked component in the sample at the detection region.

30. A method of spatially separating at least first and second components in a sample in a microfluidic device comprising introducing the first and second components into a first microfluidic channel of the device in a carrier fluid comprising a spacer electrolyte solution and stacking the first and second components by isotachophoresis between a leading electrolyte solution and a trailing electrolyte solution, wherein the spacer electrolyte solution comprises ions

which have an intermediate mobility in an electric field between the mobility of the ions present in the leading and trailing electrolyte solutions and wherein the spacer electrolyte solution comprises at least one of the following spacer ions MOPS, MES, Nonanoic acid, D-Glucuronic acid, Acetylsalicyclic acid, 4-Ethoxybenzoic acid, Glutaric acid, 3-Phenylpropionic acid, Phenoxyacetic acid, Cysteine, hippuric acid, p-hydroxyphenylacetic acid, isopropylmalonic acid, itaconic acid, citraconic acid, 3,5-dimethylbenzoic acid, 2,3-dimethylbenzoic acid, p-hydroxycinnamic acid, and 5-br-2,4-dihydroxybenzoic acid, and wherein the first component comprises a DNA-antibody conjugate and the second component comprises a complex of the DNA-antibody conjugate and an analyte.

31. The method of claim 30, wherein the complex of the DNA-antibody conjugate and an analyte is further complexed with a second antibody, Fab' antibody fragment, receptor, affinity peptide, or aptamer.

32. The method of claim 30, wherein the DNA-antibody conjugate is labeled with a fluorescent dye, an enzyme, a chemiluminescent label, or a phosphorescent label.

33. The method of claim 31, wherein the second antibody, Fab' antibody fragment, receptor, affinity peptide, or aptamer is labeled with a fluorescent dye, an enzyme, a chemiluminescent label, or a phosphorescent label.

34. The method of claim 30, wherein the carrier solution includes Tris buffer or Bis-Tris buffer and at least one of the following additional components: BSA, Tween or other carrier proteins or surfactants.

35. The method of claim 30, wherein the stacking by isotachophoresis is performed in a gel contained within the first microfluidic channel which has a concentration of between about 0.1 and 3.0%.

36. The method of claim 35, wherein the gel comprises polyacrylamide gel, polyethylene glycol (PEG), polyethyleneoxide (PEO), a co-polymer of sucrose and epichlorohydrin, polyvinylpyrrolidone (PVP), hydroxyethylcellulose (HEC), poly-N,N-dimethylacrylamide (pDMA), or an agarose gel.

37. The method of claim 30, further comprising separating the first and/or second components into additional separated components by capillary electrophoresis in the first microchannel or a second microchannel fluidly coupled to the first microchannel.

38. The method of claim 30, wherein the spacer electrolyte solution comprises MES at a pH of about 8.

39. The method of claim 30, wherein the spacer electrolyte solution comprises Nonanoic acid at a pH of about 8.

40. The method of claim 30, wherein the spacer electrolyte solution comprises Glutaric acid at a pH of about 8.

41. The method of claim 30, wherein the spacer electrolyte solution comprises D-Glucuronic acid at a pH of about 8.

42. The method of claim 31, wherein the DNA-antibody conjugate is labeled with a fluorescent dye, an enzyme, a chemiluminescent label, or a phosphorescent label.

43. A method of separating a first component of interest from at least one second component in a sample comprising:

stacking the first and second components in a first channel segment;

flowing the stacked second component through a second channel segment fluidly coupled to the first channel segment at an intersection;

measuring a voltage signal profile at or near the intersection which corresponds to the first and/or second stacked components, wherein the voltage signal profile includes at least three distinct voltage slope transitions which are separated in time; and,

applying an electric field or a pressure differential along a third channel segment which is fluidly coupled to said first channel segment at the intersection when the last in time voltage slope transition is detected, thereby introducing the stacked first component into the third channel segment.

44. The method of claim 43, wherein said stacking comprises introducing into the first channel segment a leading electrolyte buffer solution, a trailing electrolyte buffer solution, and a spacer electrolyte buffer solution between the leading and trailing electrolyte

solutions wherein the spacer buffer solution comprises ions which have an intermediate mobility in an electric filed between the mobility of the ions present in the leading and trailing electrolyte solutions.

45. The method of claim 44, wherein the spacer buffer solution comprises at least one of the following spacer ions: MOPS, MES, Nonanoic acid, D-Glucuronic acid, Acetylsalicyclic acid, 4-Ethoxybenzoic acid, Glutaric acid, 3-Phenylpropionic acid, Phenoxyacetic acid, Cysteine, hippuric acid, p-hydroxyphenylacetic acid, isopropylmalonic acid, itaconic acid, citraconic acid, 3,5-dimethylbenzoic acid, 2,3-dimethylbenzoic acid, p-hydroxycinnamic acid, and 5-br-2,4-dihydroxybenzoic acid, and wherein the first component comprises a DNA-antibody conjugate and the second component comprises a complex of the DNA-antibody conjugate and an analyte.

46. The method of claim 45, wherein the complex of the DNA-antibody conjugate and an analyte is further complexed with a second antibody, Fab' antibody fragment, receptor, affinity peptide, or aptamer.

47. The method of claim 45, wherein the DNA-antibody conjugate is labeled with a fluorescent dye, an enzyme, a chemiluminescent label, or a phosphorescent label.

48. The method of claim 46, wherein the second antibody, Fab' antibody fragment, receptor, affinity peptide, or aptamer is labeled with a fluorescent dye, an enzyme, a chemiluminescent label, or a phosphorescent label.

49. The method of claim 45, wherein the spacer buffer solution includes Tris buffer or Bis-Tris buffer and at least one of the following additional components: BSA, Tween or other carrier proteins or surfactants.

50. The method of claim 45, wherein the stacking by isotachophoresis is performed in a gel contained within the first microfluidic channel which has a concentration of between about 0.1 and 3.0%.

51. The method of claim 50, wherein the gel comprises polyacrylamide gel, polyethylene glycol (PEG), polyethyleneoxide (PEO), a co-polymer of sucrose and

epichlorohydrin, polyvinylpyrrolidone (PVP), hydroxyethylcellulose (HEC), poly-N,N-dimethylacrylamide (pDMA), or an agarose gel.

52. The method of claim 44, further comprising separating the first and/or second components into additional separated components by capillary electrophoresis in the first microchannel or a second microchannel fluidly coupled to the first microchannel.

53. The method of claim 44, wherein the spacer buffer solution comprises MES at a pH of about 8.

54. The method of claim 44, wherein the spacer buffer solution comprises Nonanoic acid at a pH of about 8.

55. The method of claim 44, wherein the spacer buffer solution comprises Glutaric acid at a pH of about 8.

56. The method of claim 44, wherein the spacer buffer solution comprises D-Glucuronic acid at a pH of about 8.

57. The method of claim 44, wherein the spacer buffer solution comprises ions which have an intermediate mobility in an electric filed between a mobility of the first and second components.

58. The method of claim 43, wherein the method is used to distinguish and compare different levels of various fractions of AFP

Fig. 1A

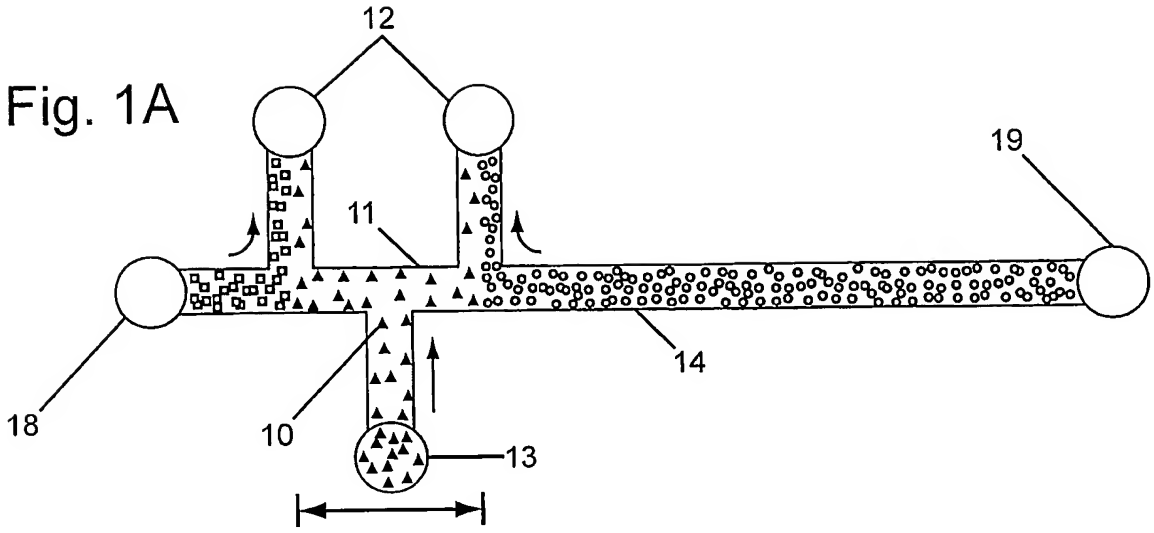


Fig. 1B

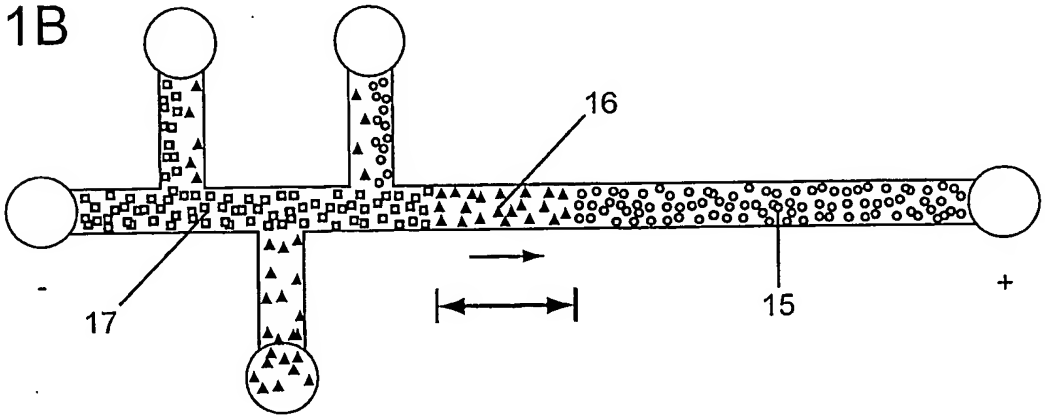
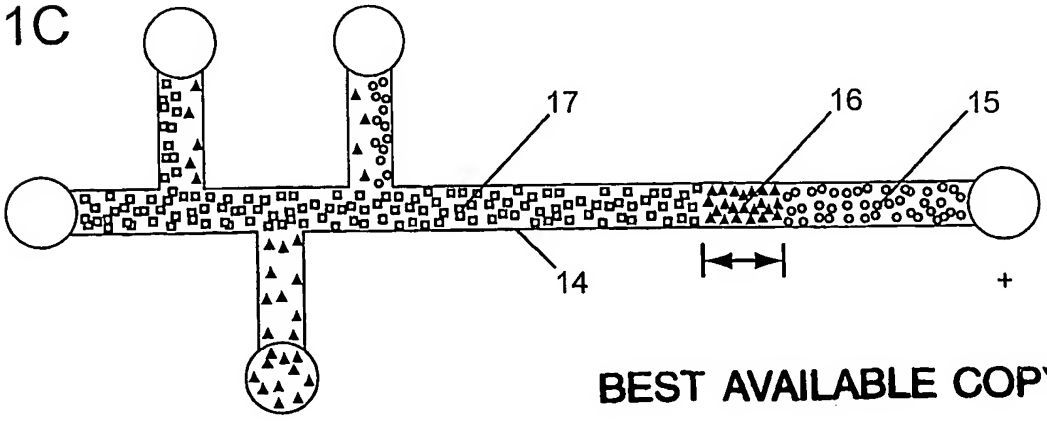


Fig. 1C



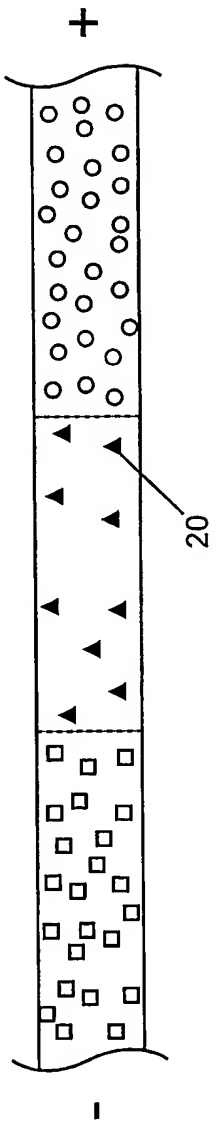


Fig. 2A

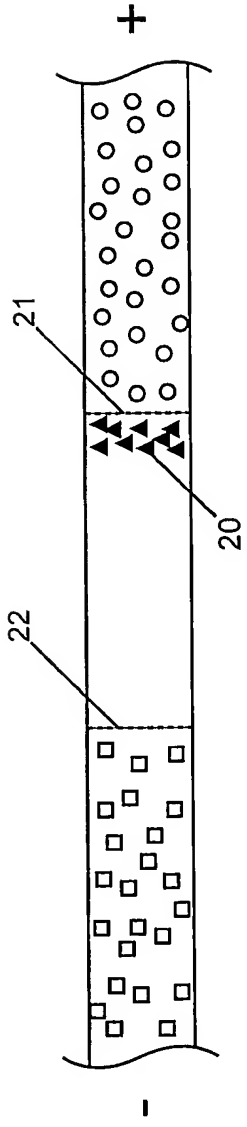


Fig. 2B

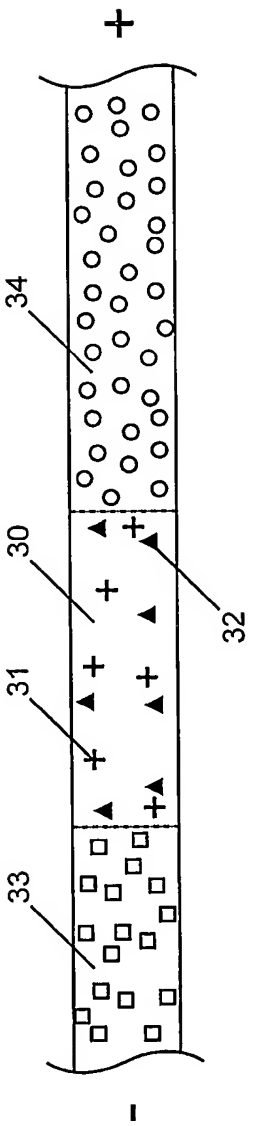


Fig. 3A

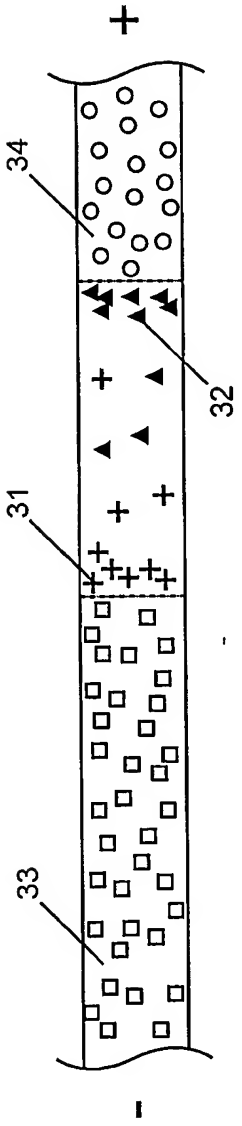


Fig. 3B

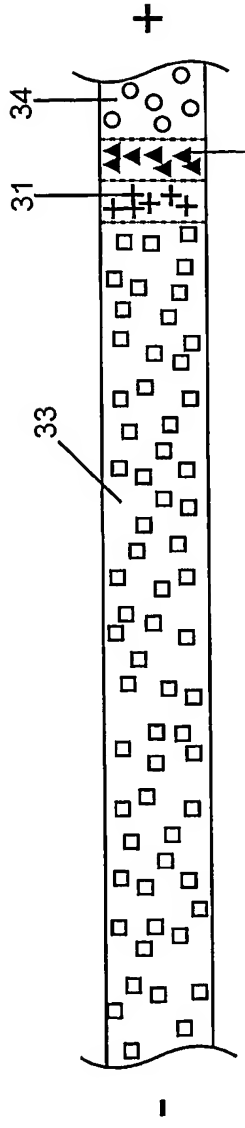


Fig. 3C

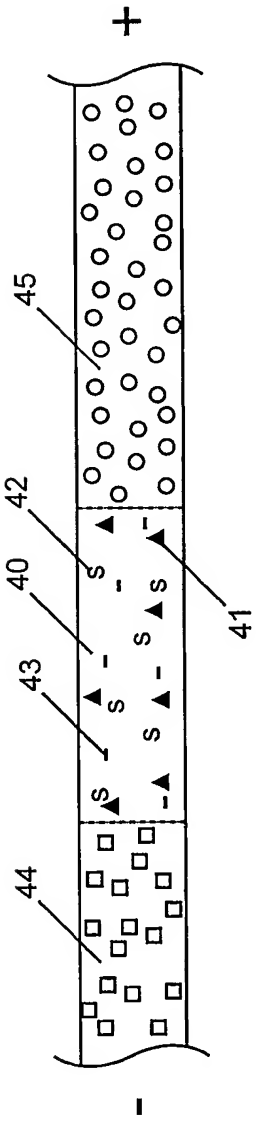


Fig. 4A

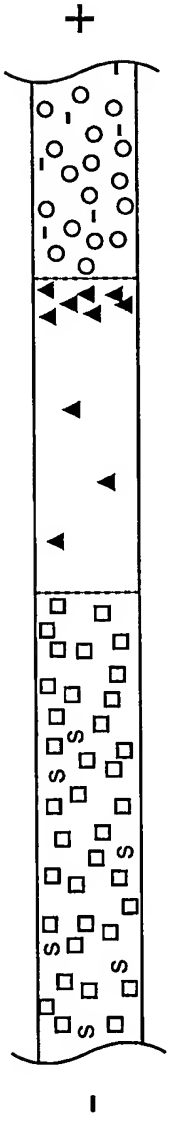


Fig. 4B

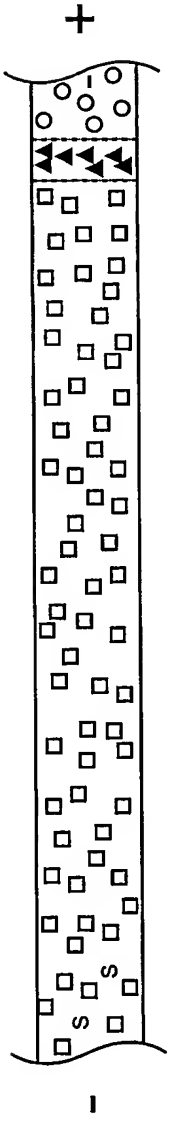


Fig. 4C

Fig. 5A

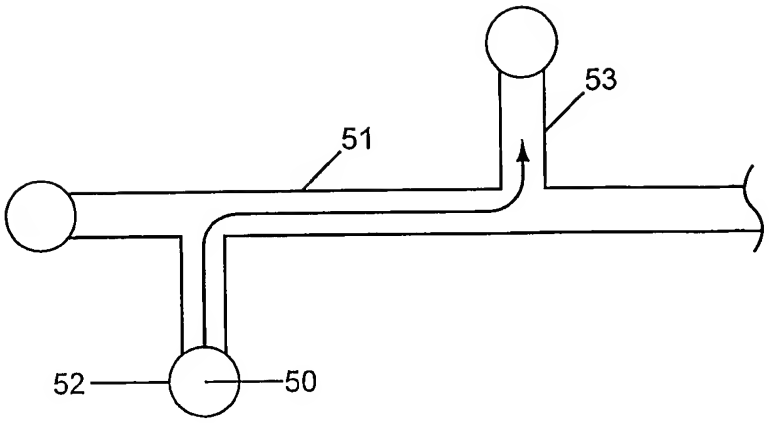


Fig. 5B

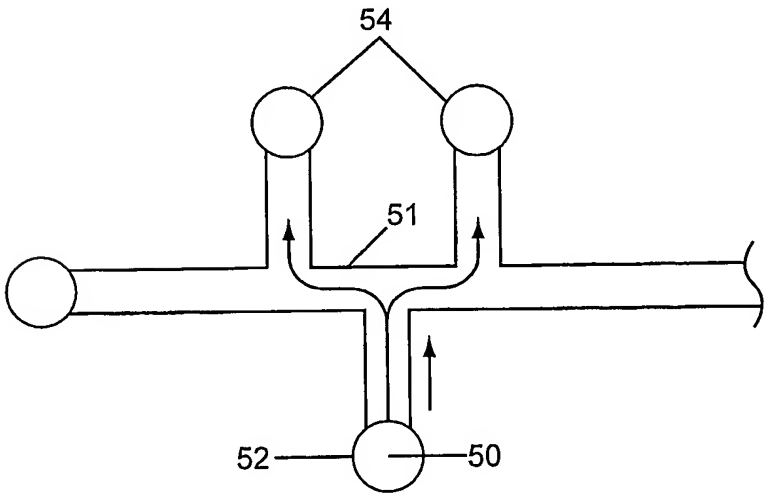


Fig. 5C

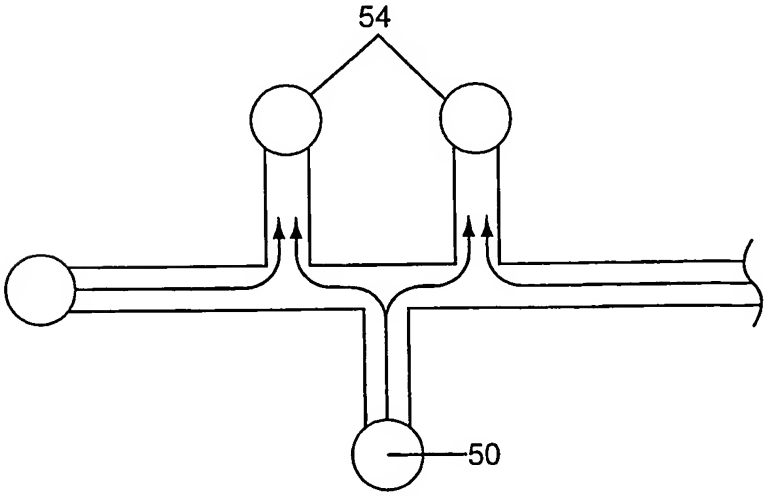


Fig. 6A

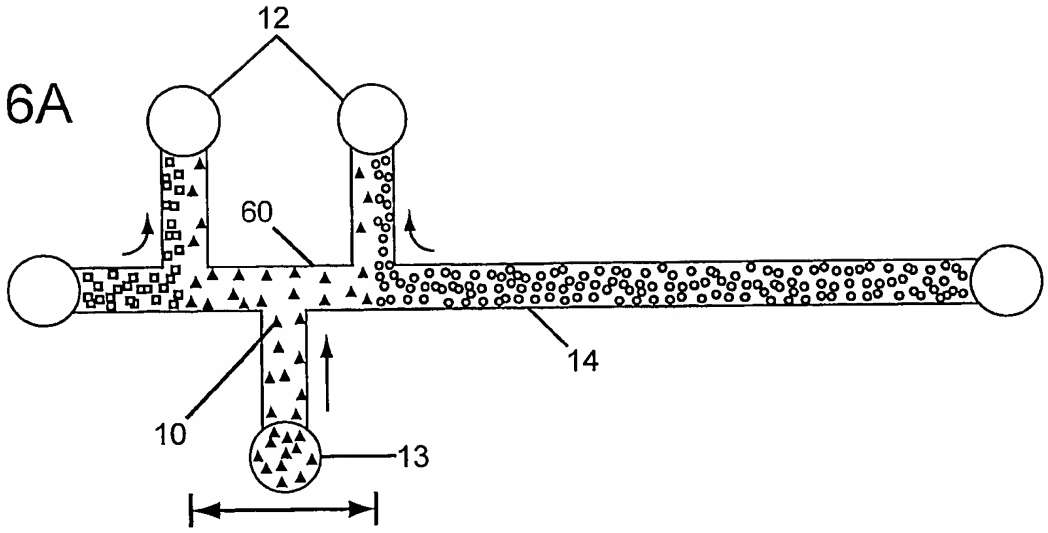


Fig. 6B

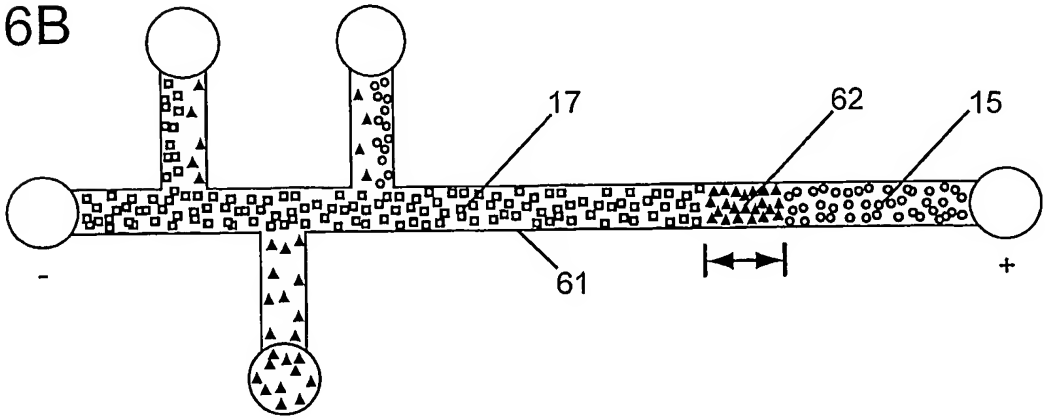


Fig. 6C

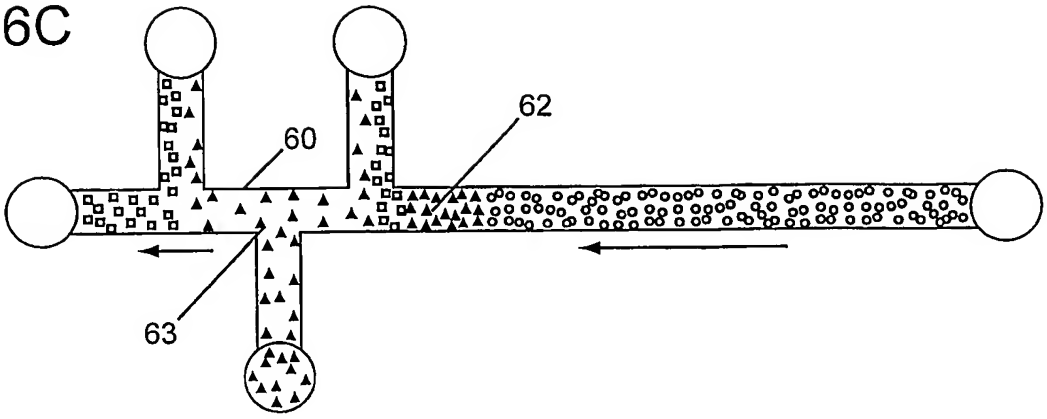


Fig. 6D

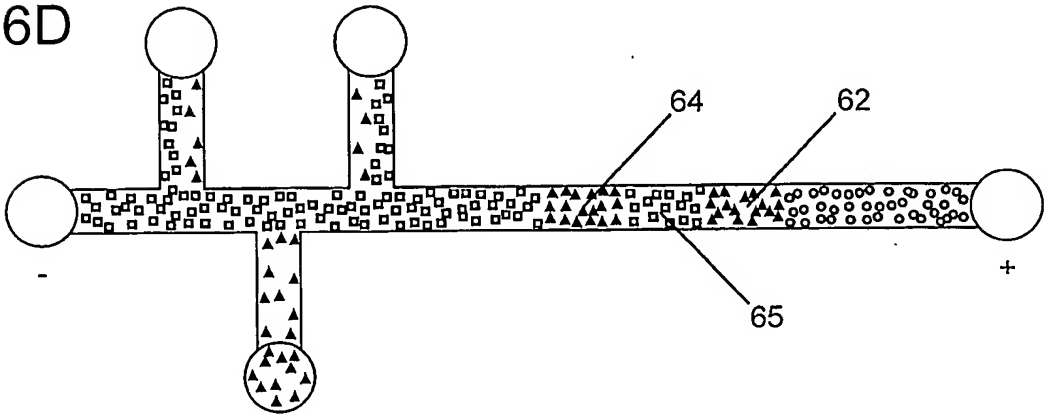


Fig. 6E

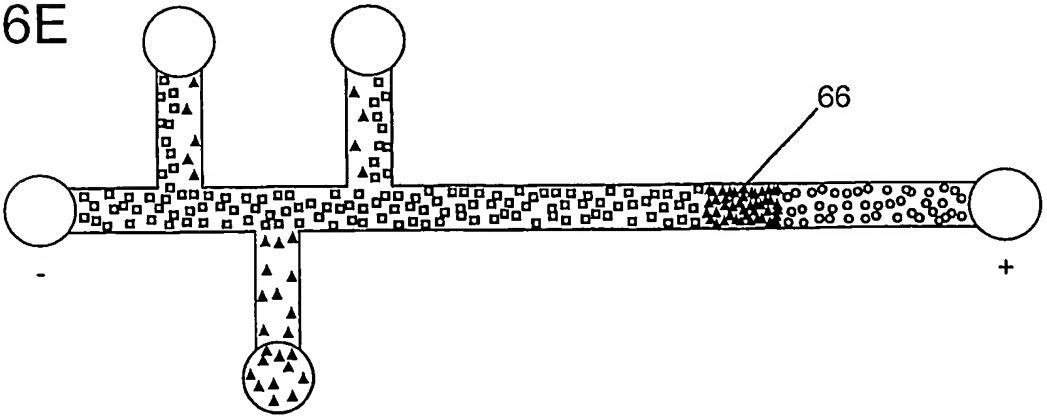


Fig. 7A

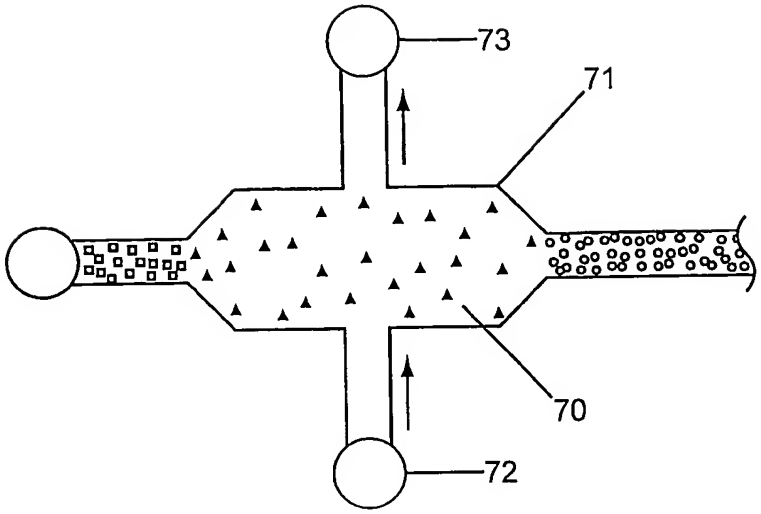


Fig. 7B

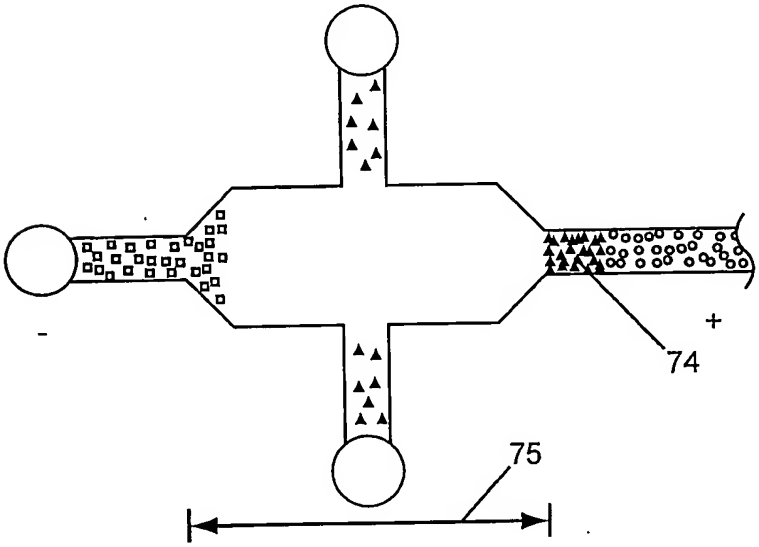
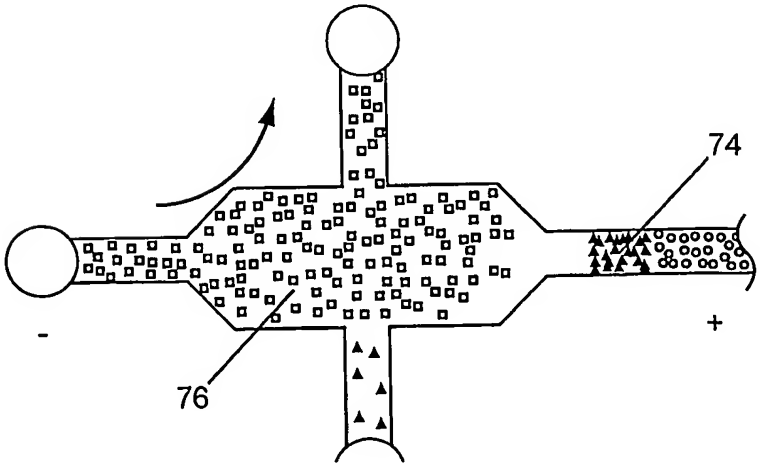


Fig. 7C



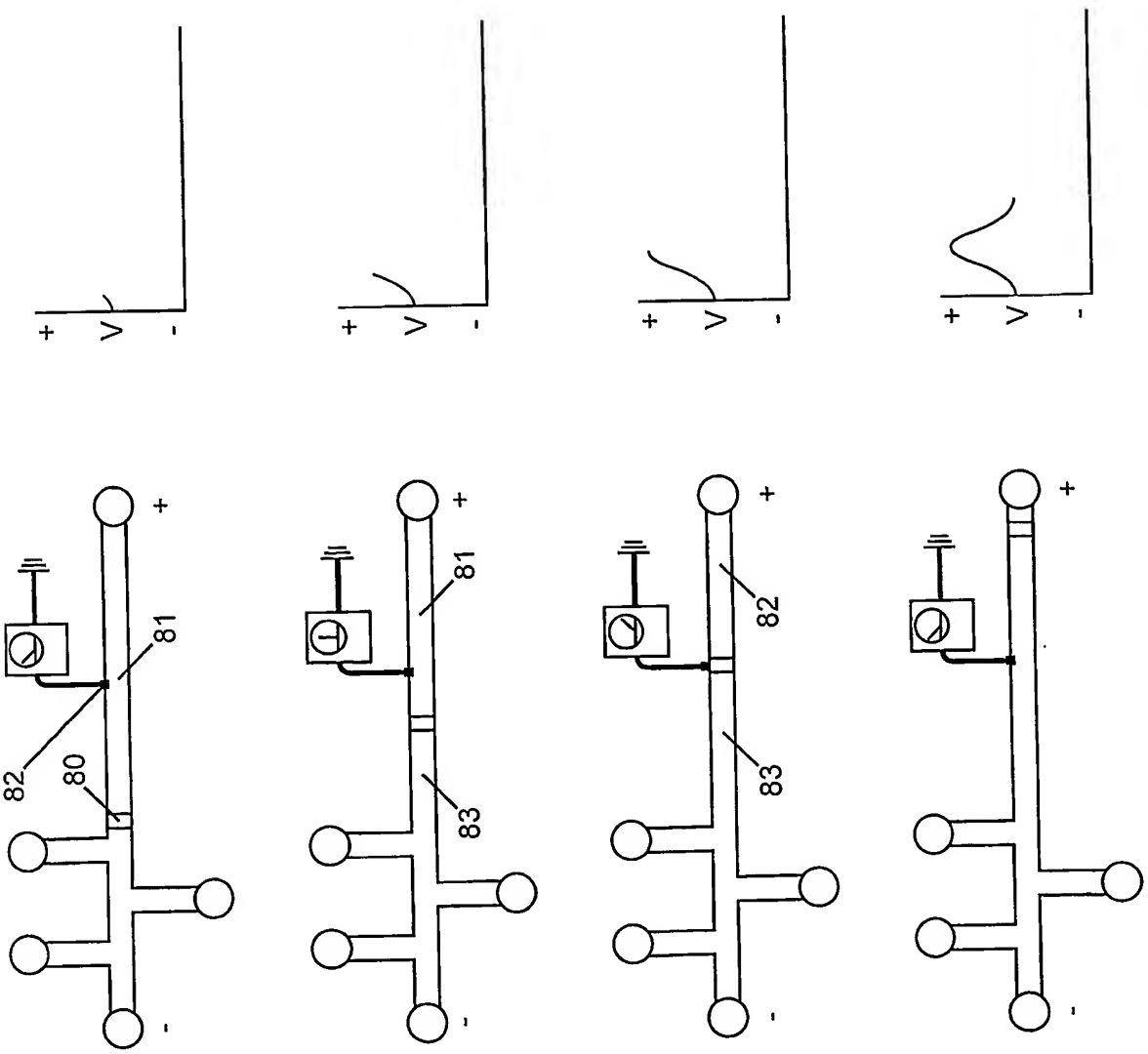


Fig. 8A

Fig. 8B

Fig. 8C

Fig. 8D

Fig. 9A

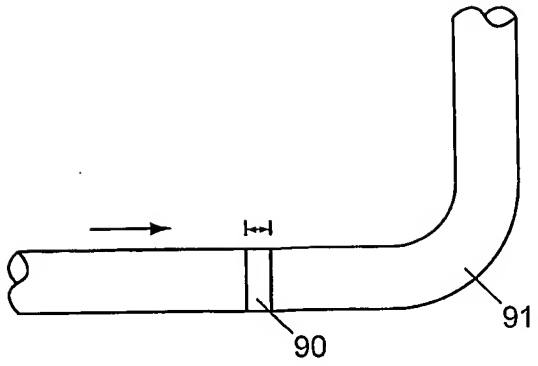


Fig. 9B

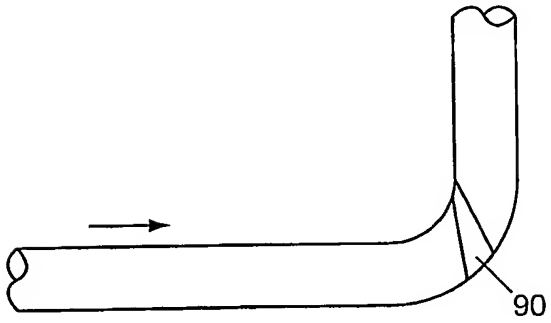


Fig. 9C

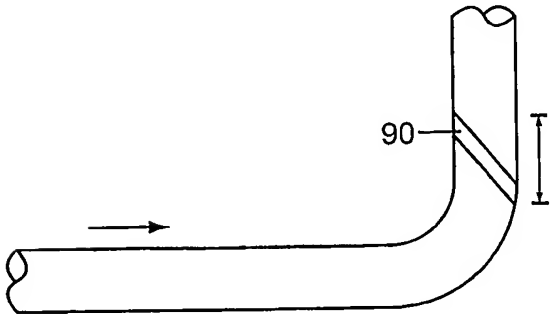


Fig. 9D

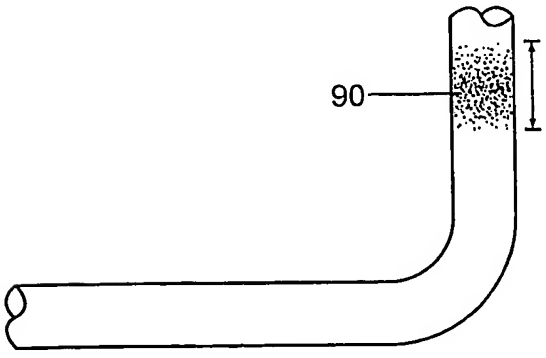


Fig. 10A

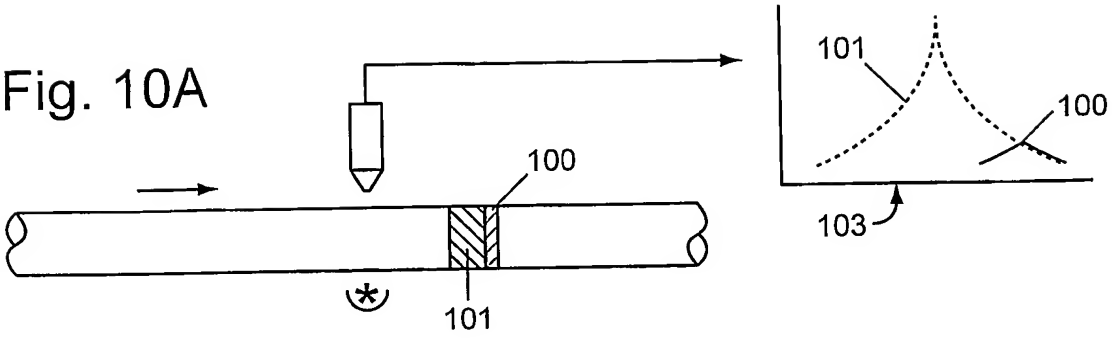


Fig. 10B

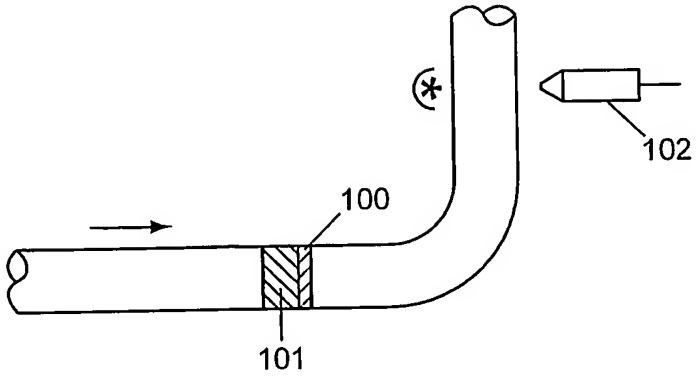


Fig. 10C

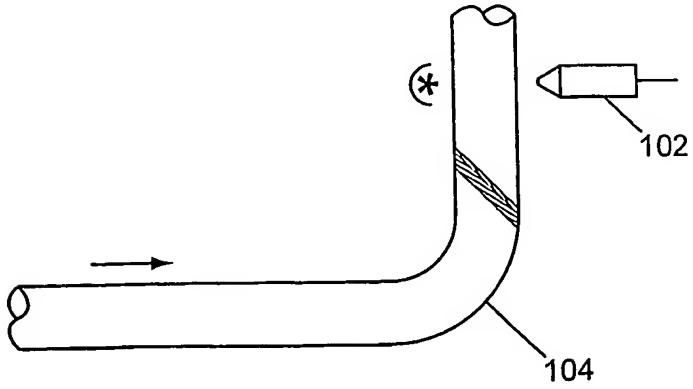


Fig. 10D

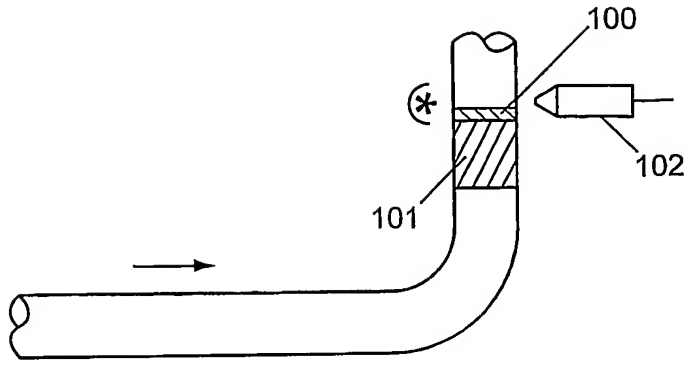


Fig. 10E

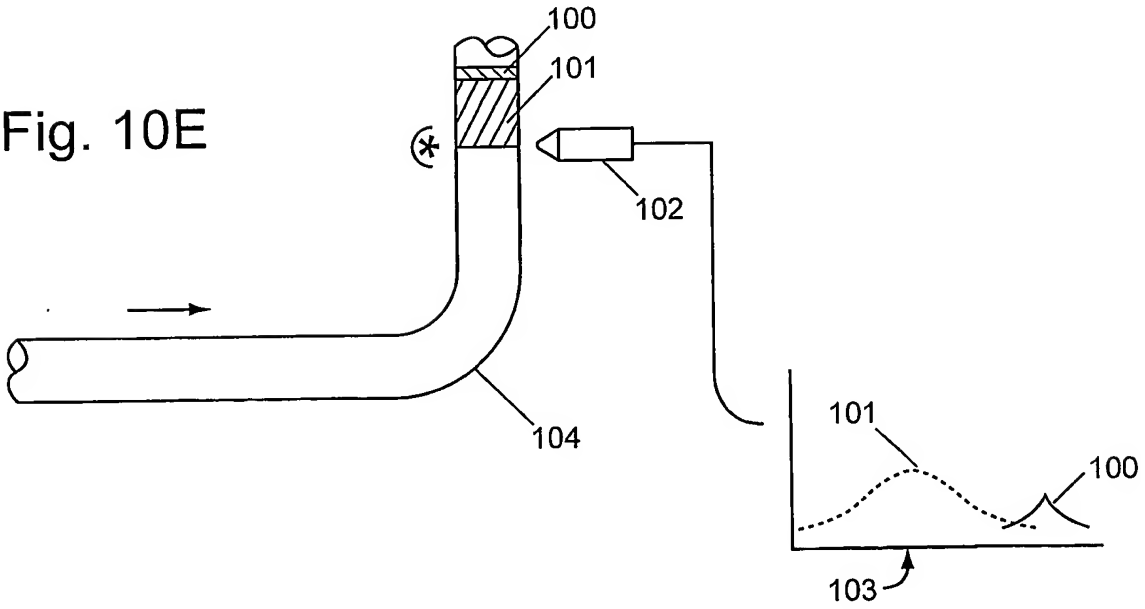


Fig. 11A

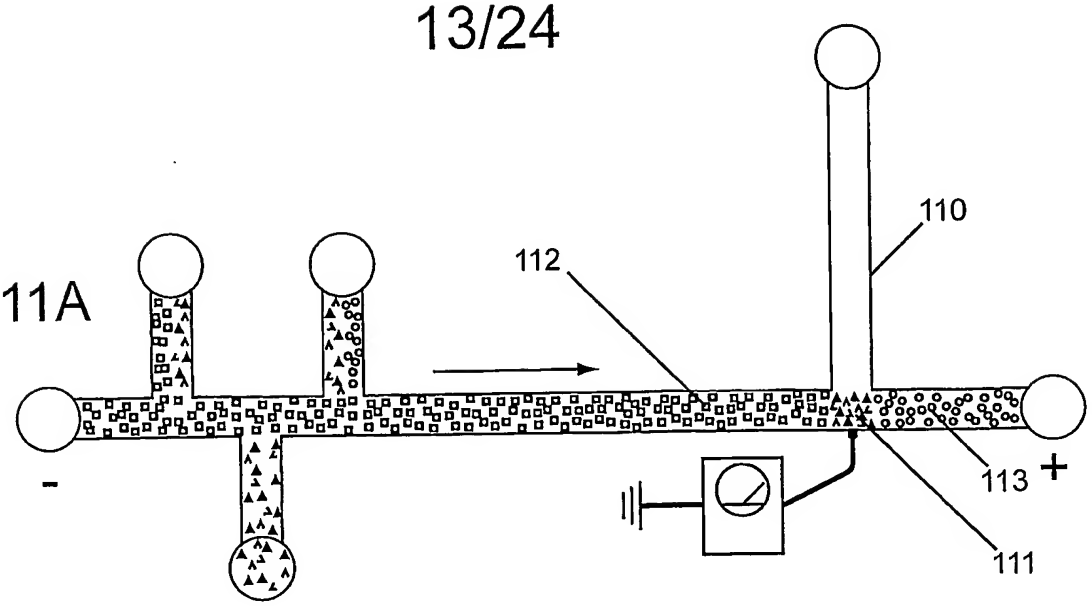


Fig. 11B

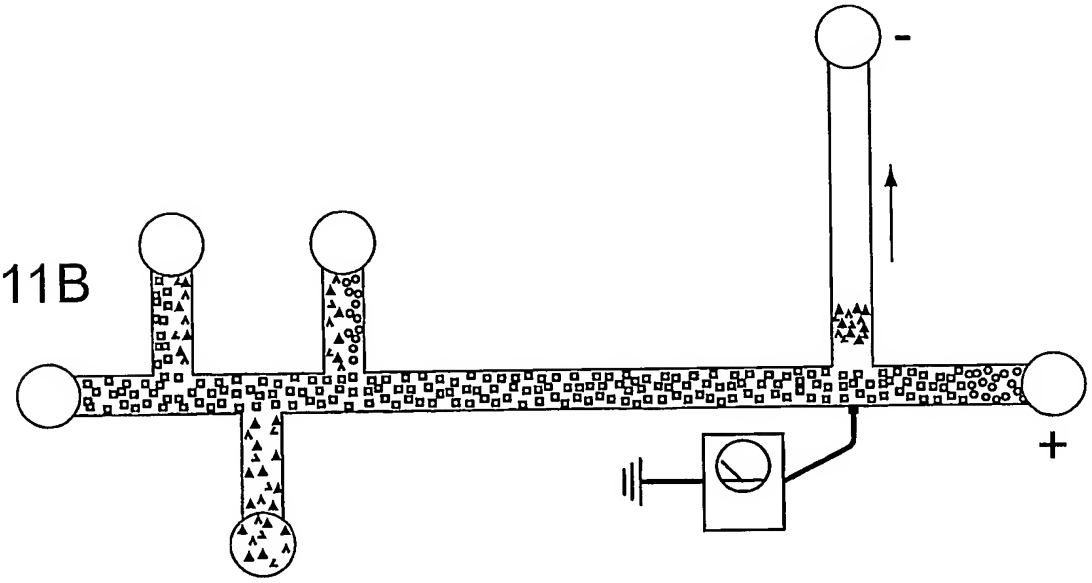
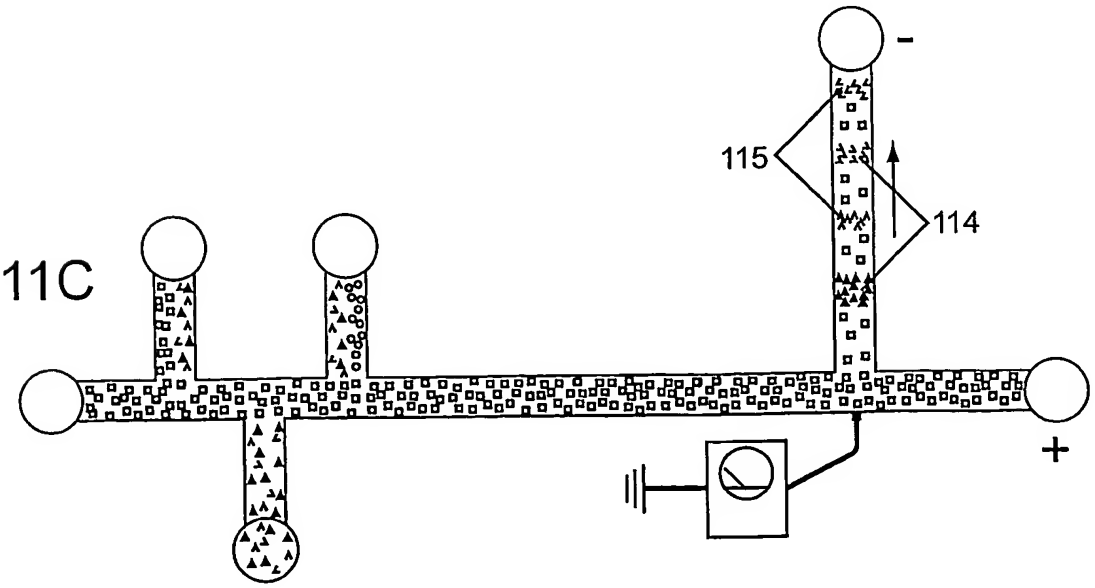


Fig. 11C



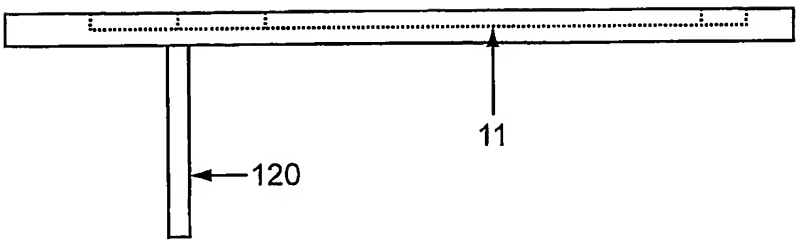


Fig. 12A

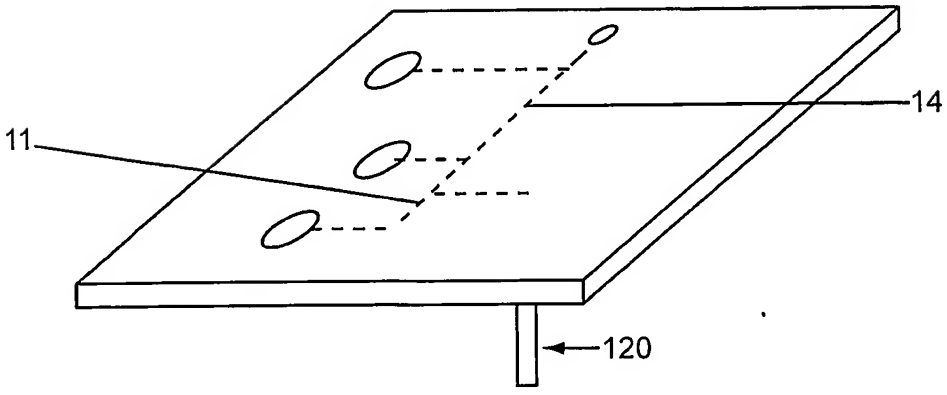


Fig. 12B

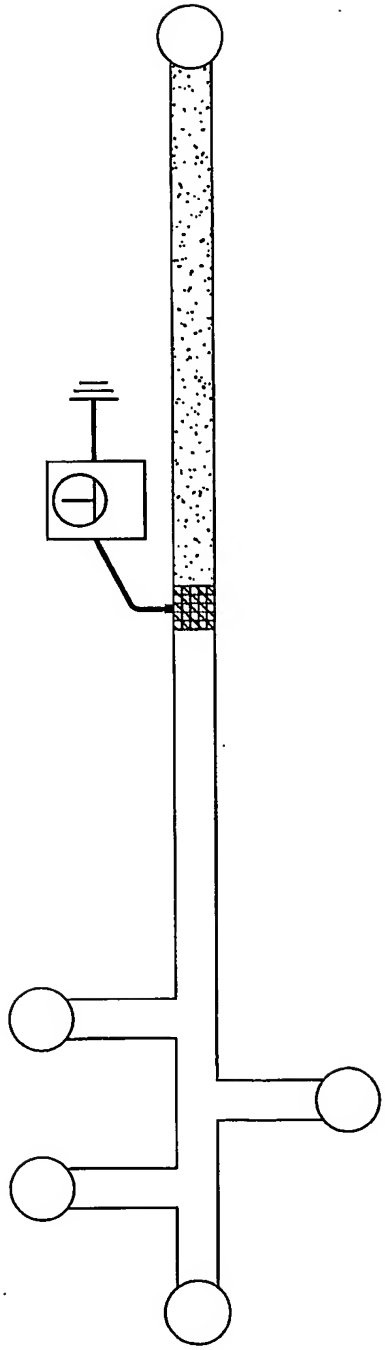


Fig. 13A

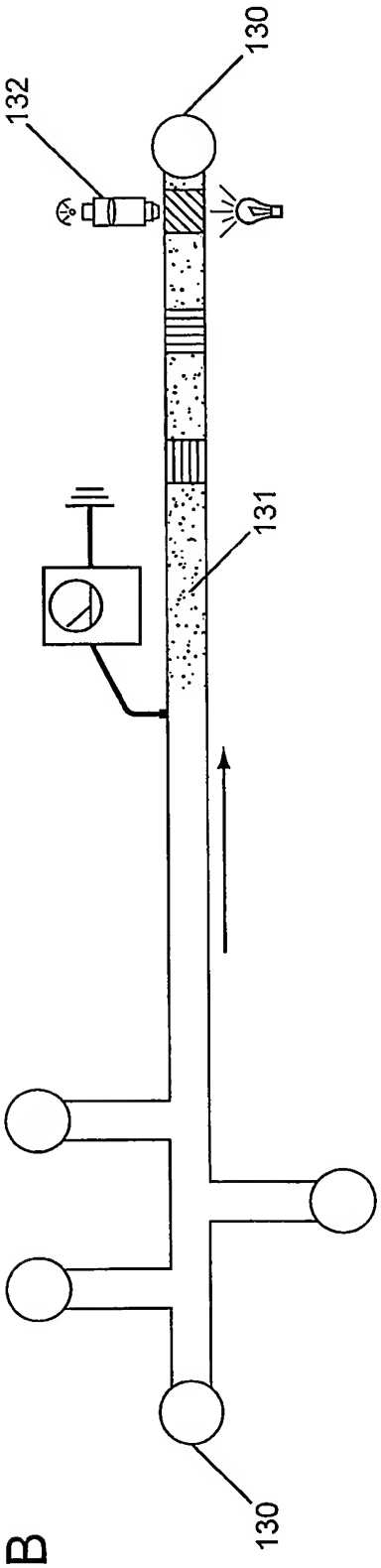


Fig. 13B

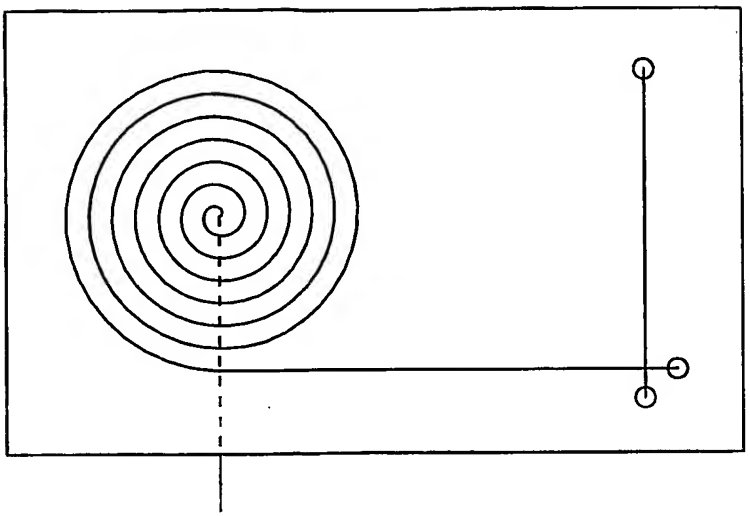


Fig. 14A

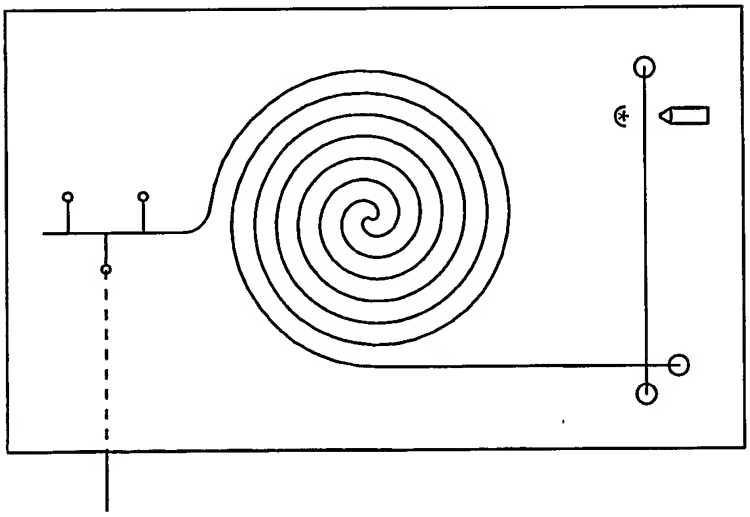


Fig. 14B

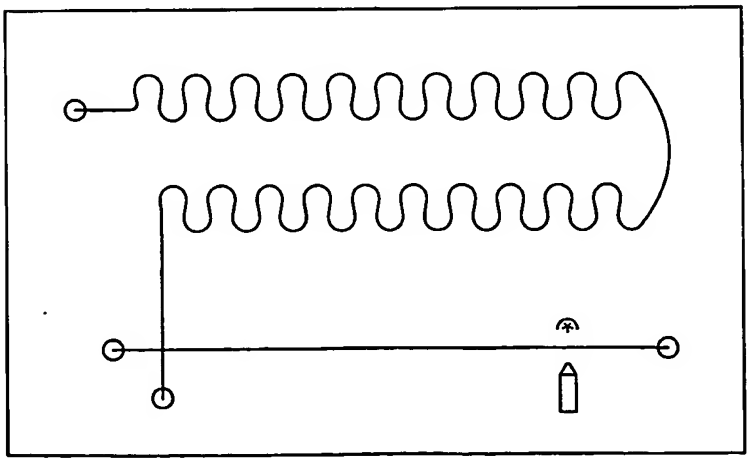


Fig. 14C

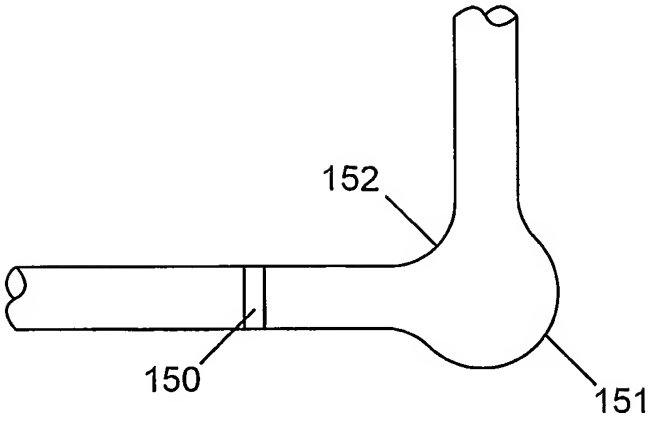


Fig. 15A

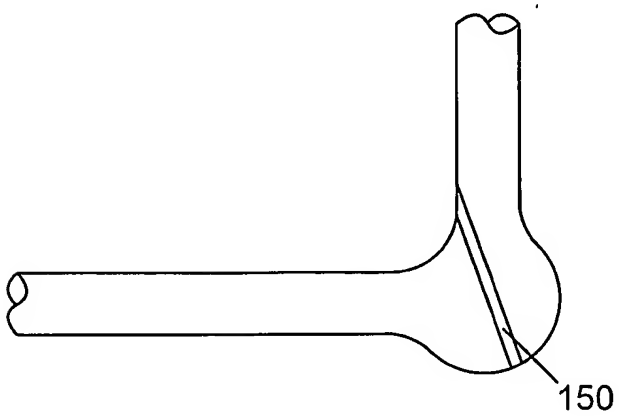


Fig. 15B

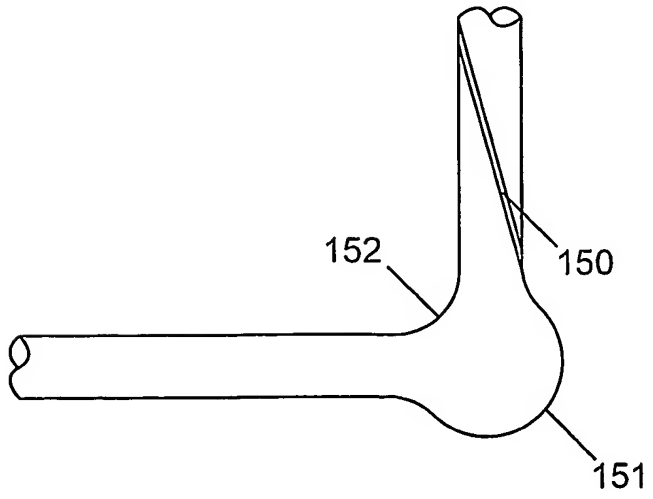


Fig. 15C

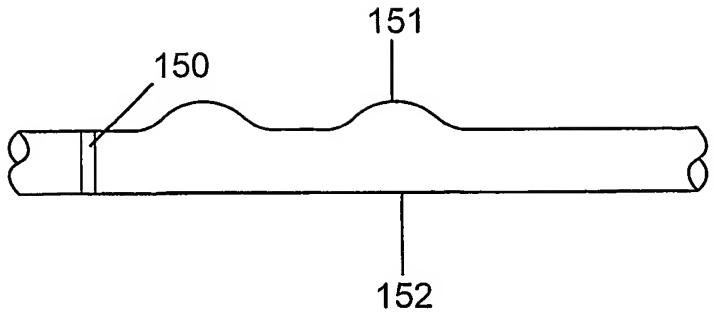


Fig. 16A

Fig. 16B

Fig. 16C

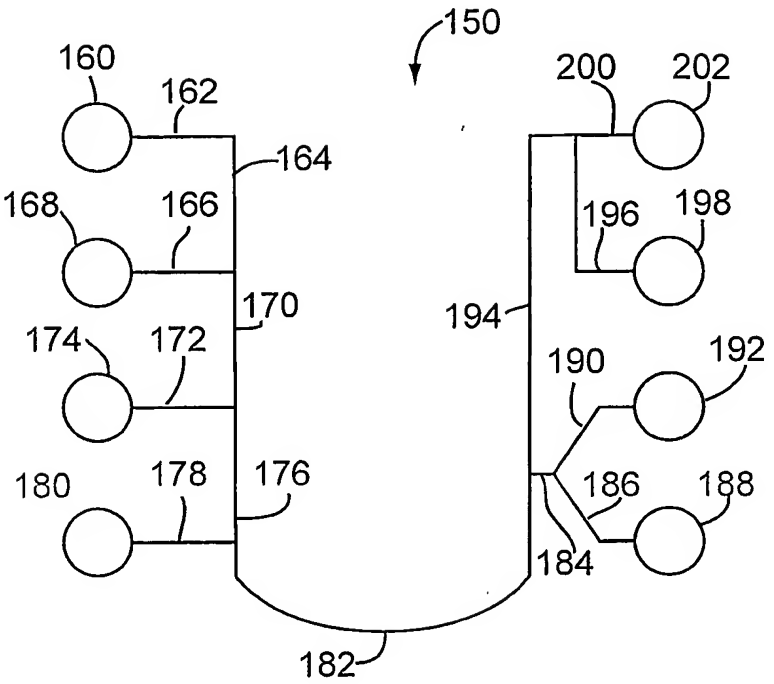


Fig. 17

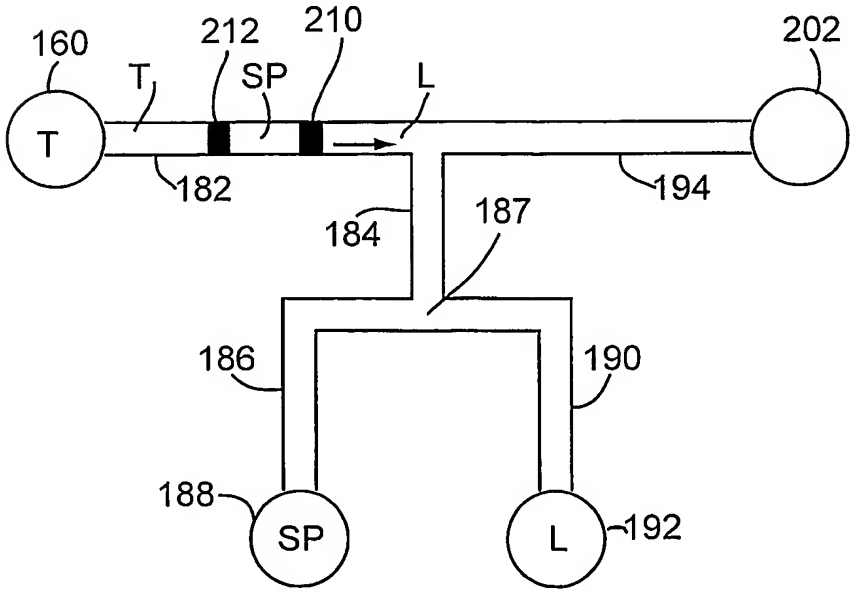


Fig. 18A

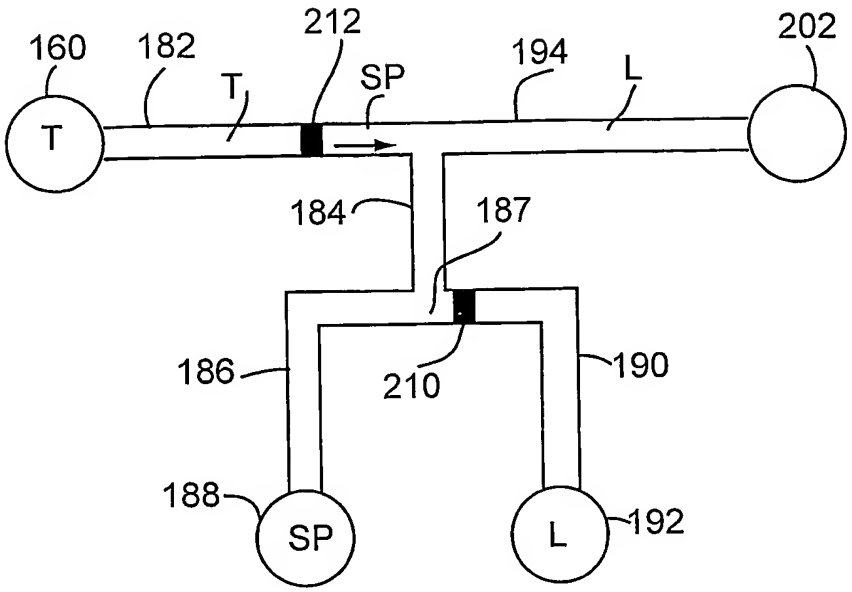


Fig. 18B

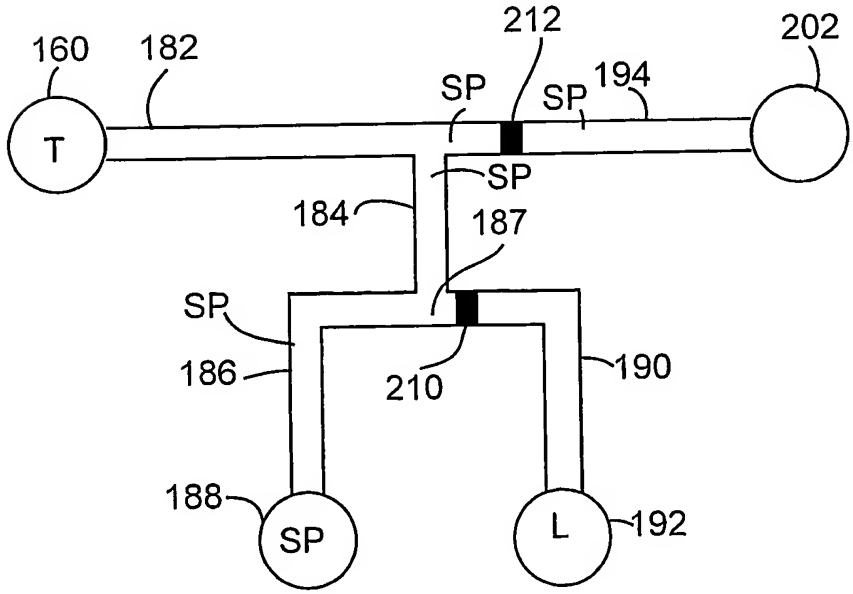


Fig. 18C

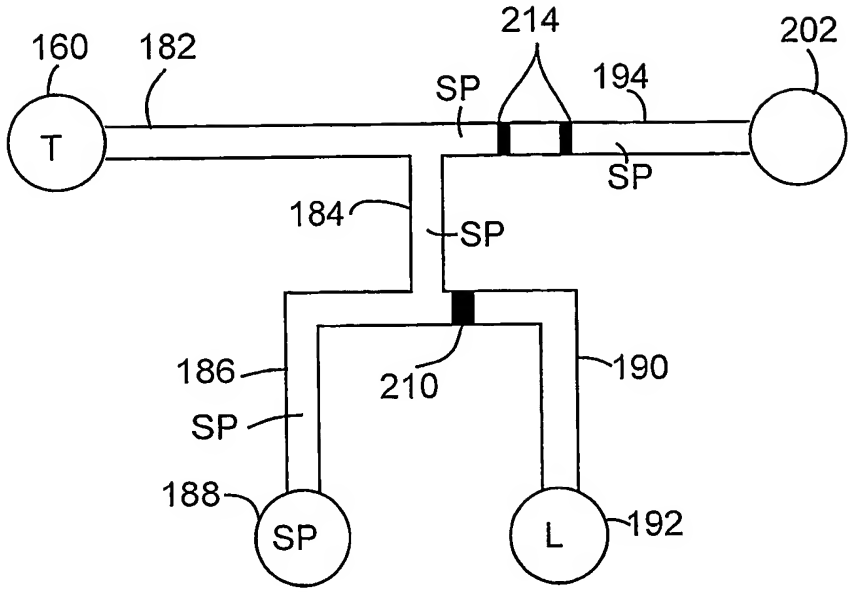


Fig. 18D

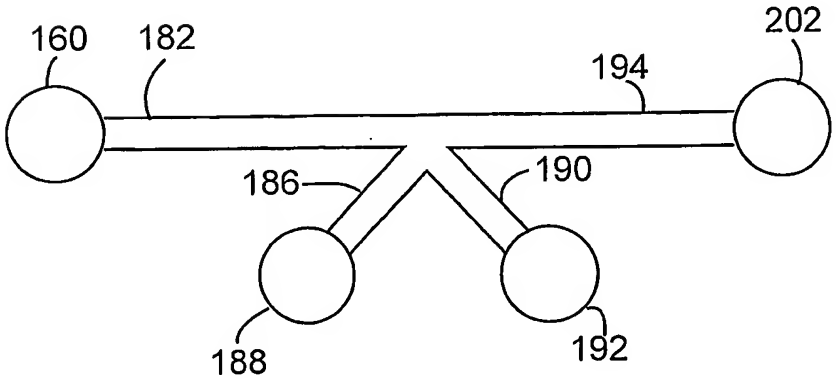


Fig. 19

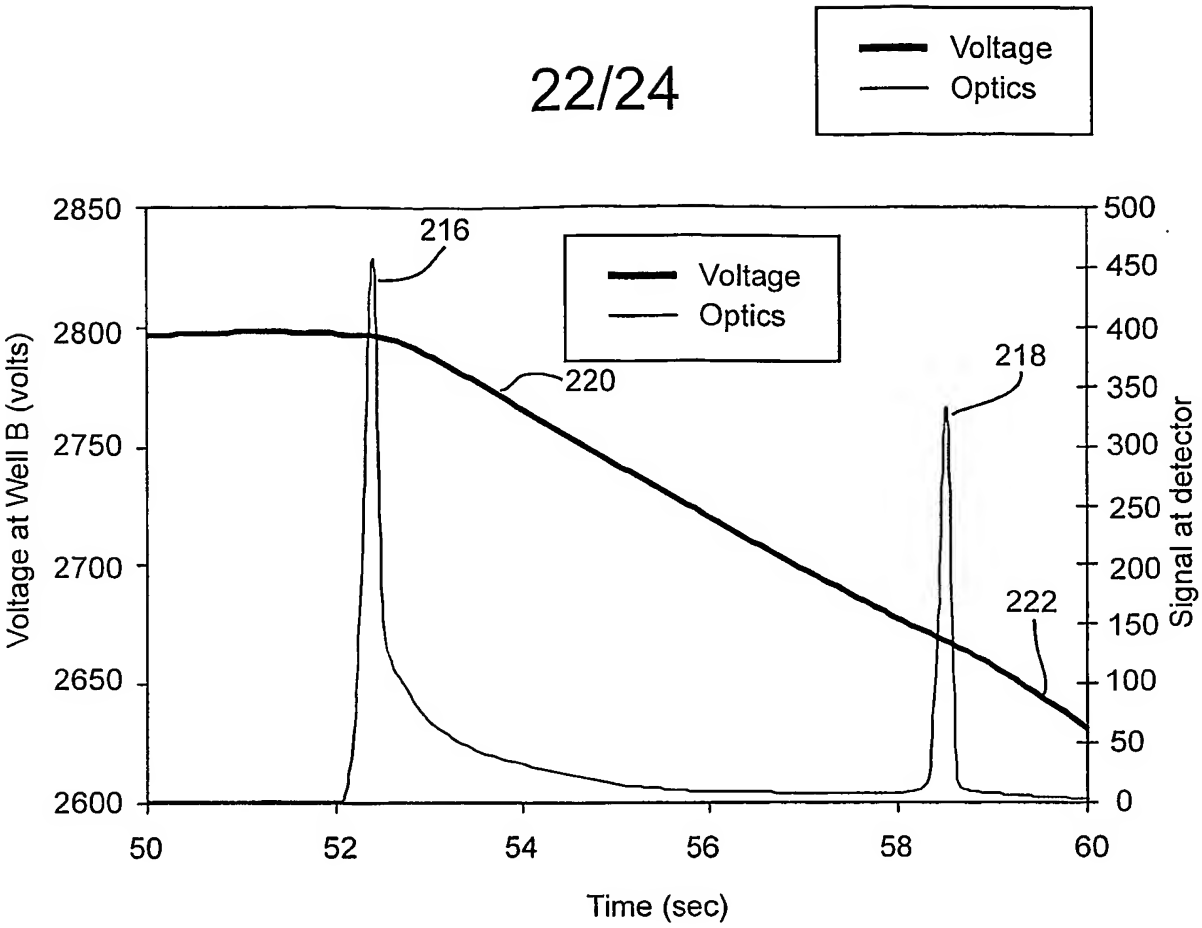


Fig. 20A

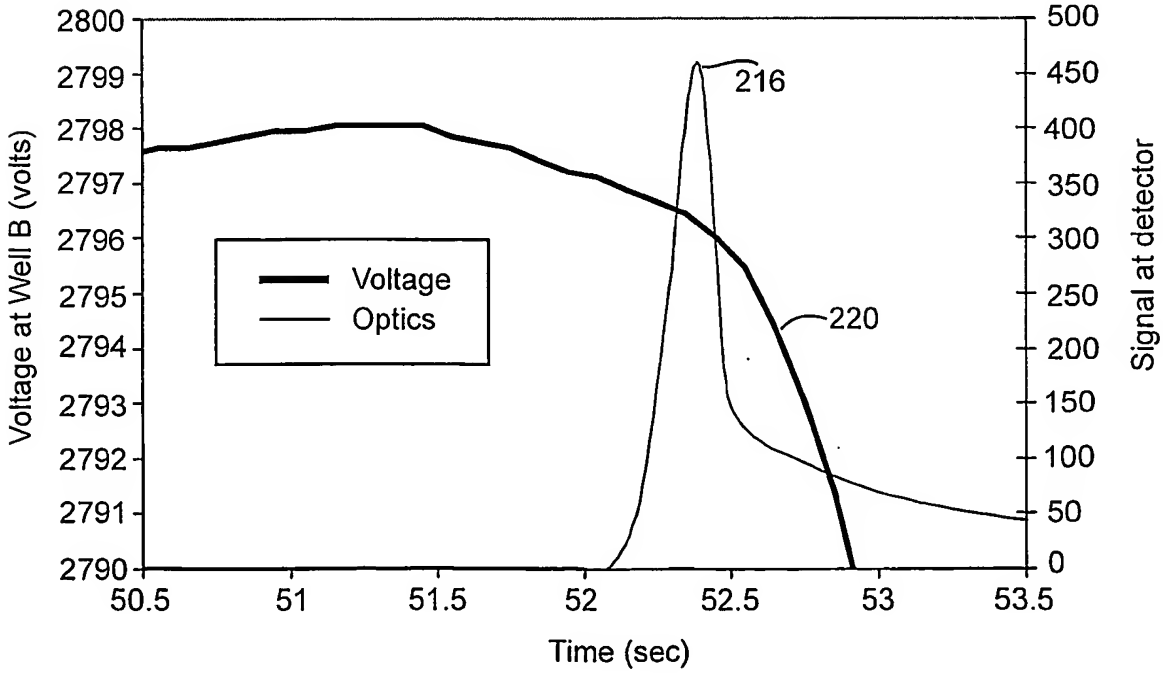


Fig. 20B

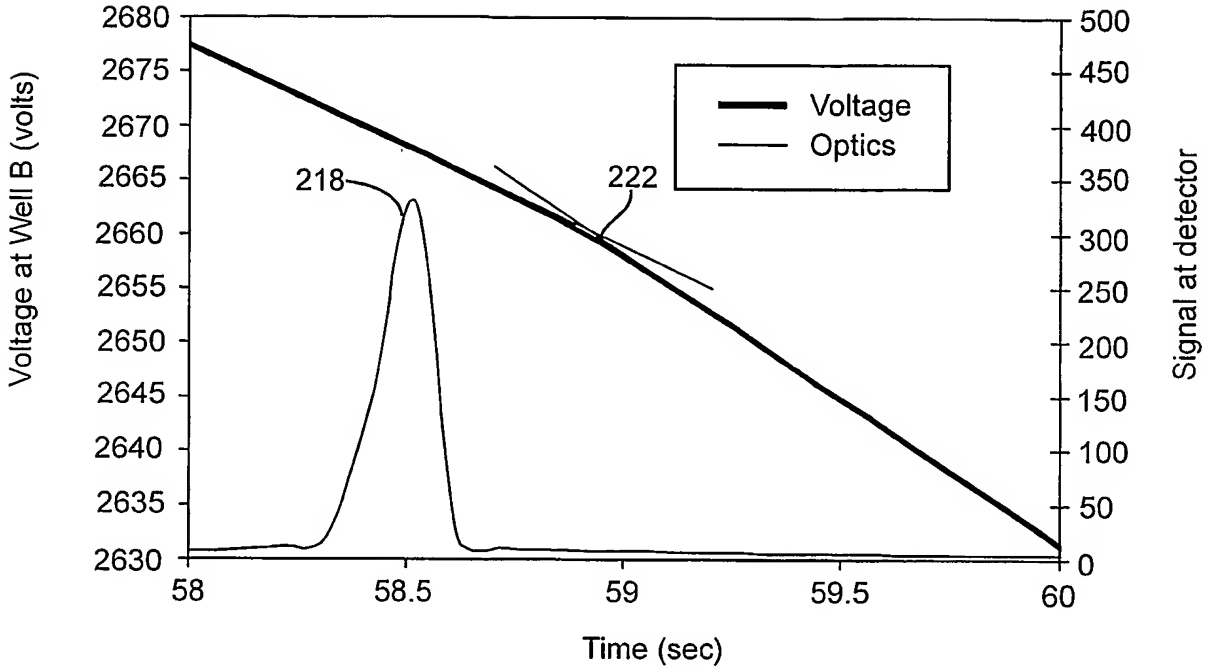

2680
2675
2670
2665
2660
2655
2650
2645
2640
2635
2630
Voltage at Well B (volts)
500
450
400
350
300
250
200
150
100
50
0
Signal at detector
58
58.5
59
59.5
60
Time (sec)
Voltage
Optics
218
222

Fig. 20C

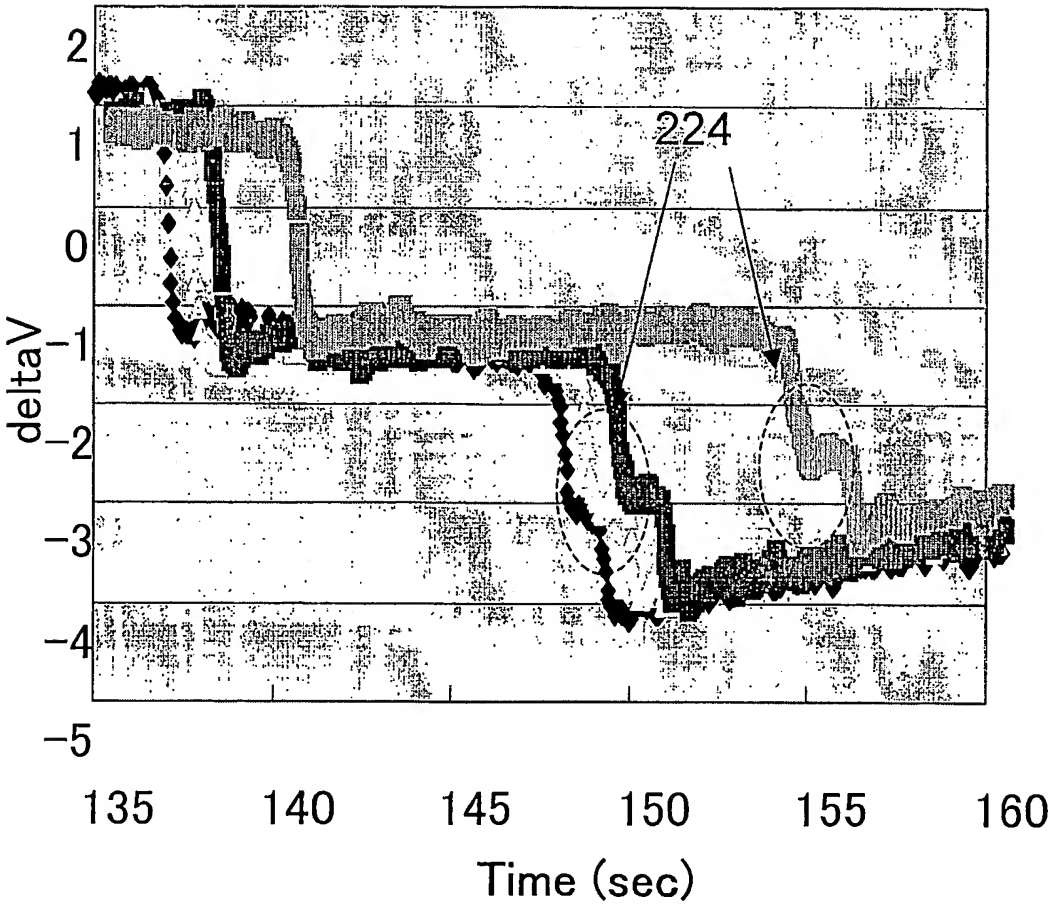


Fig. 21

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/018551

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N27/447 B01L3/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N B01L C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 199 27 535 A (MERCK PATENT GMBH ; FOERDERUNG DER SPEKTROCHEMIE U (DE)) 4 January 2001 (2001-01-04) the whole document	1,3-5, 12, 18-21, 24-29
Y		2,6-11, 13-17, 43,44, 52-58
Y	US 5 817 225 A (HINTON STEPHEN M) 6 October 1998 (1998-10-06) abstract column 1, line 42 - line 64 column 2, line 14 - line 28 column 3, line 5 - line 54	2,7-10, 13-16, 35,36

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2005	11. 03. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bravin, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/018551

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 348 633 A (KARGER BARRY L ET AL) 20 September 1994 (1994-09-20) abstract column 49 - column 60 figure 1	6,11
Y	DATABASE WPI DERWENT PUBLICATIONS LTD., LONDON, GB; 2003-058581 XP002309848 abstract & WO 02/082083 A (KAWABATA TOMOHISA ; NAKAMURA KENJI (JP); SATOMURA SHINJI (JP); WAKO PU) 17 October 2002 (2002-10-17)	17
Y	PREST J E ET AL: "Single electrode conductivity detection for electrophoretic separation systems" JOURNAL OF CHROMATOGRAPHY A, ELSEVIER SCIENCE, NL, vol. 836, no. 1, 19 March 1999 (1999-03-19), pages 59-65, XP004161556 ISSN: 0021-9673 abstract page 62, column 2, paragraph 3 figure 3	43,44, 52-58
X	US 2002/189946 A1 (WAINRIGHT ANN K ET AL) 19 December 2002 (2002-12-19) abstract paragraphs '0076!, '0079!, '0082!, '0083! figure 3	22,23
X	US 5 948 231 A (FUCHS ET AL) 7 September 1999 (1999-09-07) abstract column 1, line 47 - line 65 column 2, line 40 - line 60 column 23, line 48 - line 63	22
Y	WANG ET AL: "Immunoassays using capillary electrophoresis laser induced fluorescence detection for DNA adducts" ANALYTICA CHIMICA ACTA, vol. 500, 19 December 2003 (2003-12-19), pages 13-20, XP002318779 ELSEVIER Available online 25 June 2003 abstract	30-42

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/018551

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 536 382 A (SUNZERI ET AL) 16 July 1996 (1996-07-16) abstract column 2, line 23 - line 31 column 2, line 50 - line 52 column 5, line 58 - line 65 column 6, line 56 - column 7, line 15 column 7, line 1 - line 4 column 7, line 56 - line 57	30-42, 53-56
X	GRASS ET AL: "A new PMMA-microchip device for isotachophoresis with integrated conductivity detector" SENSORS AND ACTUATORS B, vol. 72, 10 February 2001 (2001-02-10), pages 249-258, XP002318780 ELSEVIER Available online 02 Feb 2001 abstract page 255, column 1, last paragraph figure 5b figure 8	43

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/018551

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-21, 24-29

   Methods for separating a first component from a second component and microfluidic devices therefor.

---

2. claims: 22-23

   Method for separating a first component into separated components and detecting the separated components while minimizing interference from a second component during detection.

---

3. claims: 30-42

   Method for separating a DNA-antibody conjugate and a complex DNA-antibody conjugate/analyte.

---

4. claims: 43-58

   Method for separating first and second components by detecting a particular voltage signal profile including at least three distinct voltage slope transitions.

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